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STANDARDIZATION OF AN ASSAY FOR THE QUANTIFICATION OF NUCLEAR ESTROGEN RECEPTOR

by

© BARBARA A. HEPPERLE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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OF MASTER OF SCIENCE

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FALL, 1986

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.....B.A.Hepperle.....

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This is not primarily the place where we have to be, it is the place where we are.

This is not our prison but our home. It is the road we must walk and the walking of it is called life.

Because we will walk it only once, then how important it is that we should walk it with some purpose that we can call our own.

Anon.

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Standardization of an Assay for the Quantification of Nuclear Estrogen Receptor", submitted by Barbara A. Hepperle in partial fulfilment of the requirements for the degree of Master of Science in Experimental Medicine.

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Supervisor

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Date: July 31, 1986

to

Hiro

ABSTRACT

The development of assays which measure the estrogen and progesterone receptor content of human mammary tumors has provided valuable information for the prognosis and selection of therapy for patients with breast carcinoma. The current theory of the interaction of steroids with target tissues suggests that the inclusion of the total nuclear estrogen receptor data in routine assays may further improve the accuracy of identifying those patients who may benefit from endocrine therapy.

An assay for quantifying KCl-soluble and KCl-insoluble nuclear estrogen receptors was evaluated in order to achieve a standardized method and consequently more reliable and accurate data for the estrogen receptor content in human breast cancer tissue.

The analysis of estrogen receptor was standardized by using finely powdered rat uteri as a reference tissue. The use of powdered tissue minimized inaccurate quantitative estimates of receptor content due to tissue heterogeneity.

The KCl-extraction procedure employed for this nuclear assay was found to solubilize approximately 40-50% of bound-estradiol from rat uterine nuclei. The assay was validated by comparing levels of macromolecular-bound [^{3}H]estradiol in the cytosol fraction of uteri from control rats and levels in the nuclear fraction determined by exchange after an in

vivo' injection of [^{3}H]estradiol. Scatchard analysis confirmed that the number of high-affinity estradiol-binding sites in the nuclear fraction of uteri from estradiol-primed rats was similar to the number of estradiol-binding sites in the cytosol fraction of uteri from control rats. It was concluded from these experiments that the assay could be used to measure the nuclear estrogen receptor content of human breast tumors.

Using rat uterine tissue as a reference powder, the assay was applied to 34 human mammary tumors. As well as assaying the tumors for the presence of nuclear estrogen receptors, the relationships between cytosolic estrogen receptors, total nuclear estrogen receptors, and cytoplasmic progesterone receptors was studied.

None of the human breast tumor samples analyzed in this preliminary study were found to contain KCl-insoluble nuclear estrogen receptors. Both nuclear estrogen receptors and cytosolic progesterone receptors were found more frequently in tumors with higher concentrations of cytosolic estrogen receptors, but given the small sample size, there appeared to be no significant relationship between concentrations of either nuclear estrogen receptors or cytosolic progesterone receptors and cytosolic estrogen receptors.

There appeared to be a positive relationship between age and the incidence of tumors containing all three

receptors. The low incidence of nuclear estrogen receptors in tumors from premenopausal women suggests that low cytosolic steroid-binding activity is not due to binding of receptors by endogenous estrogens and may be due to other factors.

One of twelve tumors that lacked cytosolic estrogen receptors contained both nuclear estrogen receptors and cytosolic progesterone receptors. This finding may explain why a small proportion of cytosolic estrogen receptor-negative tumors consistently respond to endocrine therapy.

Also, the frequency of tumors in which all three receptor populations could be measured was comparable to the actual rate of tumor regression in response to endocrine therapy. These findings suggest that the presence of both cytosolic and nuclear estrogen receptors along with cytosolic progesterone receptors in human breast tumor samples might provide a more accurate indication of hormone-sensitive tumors.

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LIST OF ABBREVIATIONS

B ..... steroid specifically bound to receptors

BSA .....bovine serum albumin

DCC .....dextran-coated charcoal

DES .....diethylstilbestrol

DTT .....dithiothreitol

EDTA.....ethylenediaminetetraacetic acid

ERc ... .cytosolic estrogen receptor

ERn .....nuclear estrogen receptor

F ..... free steroid

fmol .....femtomole

HAP .....hydroxylapatite

Kd .....dissociation constant

MTG .....monothioglycerol

NSB .....nonspecifically bound steroid

PgR .....progesterone receptor

S .....steroid

S.D. .....standard deviation

SHBG .....sex hormone binding globulin

SR* .....activated steroid-receptor complex

LIST OF DEFINITIONS

Activation: those changes in the estrogen-receptor complex which lead to increased binding of the complex to nuclei or DNA.

Deactivation (or nonactivated state): reduced capacity of the estrogen-receptor complex to bind tightly to nuclei or DNA.

Inactivation: those changes in the receptor that result in a form which is incapable of binding steroid.

Transformation: the reaction by which the 8S or 4S estrogen-receptor complex is modified to the 5S dimer and is altered from a state with fast into a state with slow dissociation kinetics.

Translocation: the movement of receptor from the cytoplasm across the nuclear membrane into the nucleus.

CHAPTER I

GENERAL INTRODUCTION

1. Human Breast Carcinoma

Breast carcinoma is the most common malignancy in women, and is the leading cause of all deaths in women aged 35-54 years (Statistics Canada, 1983). Since early detection of this disease can lead to a more favorable prognosis (Strax, 1978; Wexler, 1978), the identification of women who may be at high risk for breast cancer is essential (MacMahon et al., 1973; Morgan and Vakil, 1974).

a) Risk Factors

The etiology of breast cancer is unknown, but several factors are associated with an increased risk of developing the disease. As with all malignant diseases, the risk of breast cancer increases with increasing age (Kalache, 1981). Environmental determinants such as viral, chemical and nutritional factors are suspected as possible causes (Carroll et al., 1968, MacMahon et al., 1973, Henderson et al., 1974; Hems, 1976; Levin et al., 1981; Gaskill et al., 1979) and they may explain why the incidence of the disease is significantly higher in women of the western world than in Asian or African women (Kalache, 1981). In addition, factors in the reproductive history are important and appear to be related to estrogen production or metabolism (MacMahon

et al., 1973; Wallace et al., 1978; Henderson and Canellos, 1980; Henderson et al., 1982). The correlation between the reproductive hormones and tumorigenesis (Bittner, 1947) has led several investigators to suggest that the causes of breast cancer among premenopausal women may differ from those among postmenopausal women (de Waard et al., 1964; 1979; Craig et al., 1974; Henderson et al., 1974; Blot et al., 1977; Choi et al., 1978; Wallace et al., 1978).

b) Prognosis of Patients with Breast Cancer

The prognosis of each patient is determined by the severity of the disease. Severity is determined by evaluating the following characteristics: tumor size, attachment to overlying skin or underlying chest wall, the presence or absence of palpable lymph nodes, and distant metastases (Savlov et al., 1978). Patients with Stage I or II disease, involving a small tumor (<2 cm) with or without nodal involvment, have the best prognosis. Patients with Stage III and IV disease, a large tumor (>5 cm) and malignant lymph nodes, have the worst prognosis (Baum, 1976). With Stage IV disease, metastases occur and may involve the lungs, bones, cutaneous tissue, liver, and brain (Cutler and Myers, 1967). Metastatic disease is not curable by conventional methods and these patients have a limited life expectancy irrespective of treatment. Any involvement of the lymph nodes is considered indicative of more advanced disease, thus the degree of nodal involvement in the axilla

is currently the most important prognostic indicator in patients with early carcinoma of the breast (Fisher et al., 1975).

c) The Management of Breast Cancer

The generally accepted treatment for most breast carcinoma is mastectomy, which may or may not be accompanied by radiation (Fisher et al., 1970a; Baum, 1976). Treatment of patients with metastatic disease (Stage IV) includes chemotherapy and/or endocrine therapy. However, despite numerous clinical trials to evaluate the effectiveness of adjuvant therapy, no optimal treatment has yet been defined (Lippman, 1985).

Treatment of carcinoma of the breast by radical mastectomy (removal of the entire breast plus underlying muscles and lymph nodes, Halsted, 1907), was based on the belief that the cancer spread very slowly from one of several loci in the breast along lymphatic channels until it became trapped in the regional lymph nodes. Once the lymph nodes became saturated, the cancer then spread to the skeleton and vital organs. Later evidence indicated that tumor cells could invade the vascular system without any lymphatic involvment (Fisher, 1970b). Consequently, current surgical therapies are less severe and radical mastectomies are now seldom performed (Haagensen, 1974; Baum, 1983).

Metastatic disease is virtually incurable, and the main emphasis is given to palliative care, that is, improving the quality of, rather than the length of the patient's life (Baum, 1980). Since approximately 30% of all breast tumors depend upon steroid hormones for their growth (McGuire et al., 1975a, 1975b), patients with hormone-dependent tumors may experience relief from symptoms as well as tumor regression with some form of endocrine manipulation (Rossof, 1980). Endocrine manipulation, defined by Rossof (1980) as the removal of the sex steroid hormone producing tissue, or the addition of steroid or steroid-like sex-hormones, is currently the recommended treatment for metastatic breast carcinoma in most postmenopausal patients (DeSombre et al., 1979; Lippman, 1985). Given the fact that hormonal therapy has minimal toxicity compared to chemotherapy, it is desirable to identify those patients who could benefit from this less toxic mode of treatment.

Breast tumors considered the most likely to respond to endocrine intervention (tumor regression) have considerable amounts of cytosolic (high speed supernatant fraction of cell homogenates) estrogen and progesterone receptor proteins (McGuire et al., 1975b; Heuson et al., 1975; Block et al., 1978; Allegra et al., 1980). For patients with comparable lymph node involvement, those whose tumors contain appreciable amounts of estrogen and progesterone receptors show a lower recurrence rate and significantly longer disease-free interval following mastectomy than do

those patients whose tumors contain no or only low receptor concentrations (Jensen et al., 1975; 1976; Jensen, 1980). Consequently, in order to provide the clinician with information relevant to prognosis and selection of therapy, samples of excised tumors are routinely assayed to determine estrogen and progesterone receptor content.

2. History of Hormonal Manipulation for Human Breast Cancer

a) Introduction

The concept that endocrine manipulation could play an important role in the management of breast cancer was apparently first recognized by Albert Schinzinger in 1889 when he noted that prognosis was worse in younger women (Rossof, 1980). Schinzinger hypothesized that removal of the ovaries would cause the mammary gland to atrophy, containing the tumor within the shrinking tissue.

In 1896, George Beatson independently reported the first therapeutic ovariectomies in two premenopausal women with inoperable breast tumors and described significant tumor regression. Although his findings were confirmed by others (Boyd 1897, Gould 1897), the beneficial effects, which occured in only 30% of the patients, appeared to be transient, lasting from 6-12 months. Thus ovariectomy, for the treatment of breast cancer was not accepted as a standard procedure until many years later.

The observation that changes in the hormonal environment could influence the 'in vivo' growth rate of mouse mammary tumors (Lacassagne, 1936; Haddow et al.,1944) led Bittner (1947) to suspect that reproductive hormones were associated with tumorigenesis in humans. The assumption that decreasing circulating levels of hormones could induce regression of hormone-dependent tumors renewed interest for endocrine ablative procedures such as ovariectomy (Pearson et al., 1953; 1954), adrenalectomy (Huggins and Bergenstal, 1952; Pearson and Ray, 1959a) and hypophysectomy (Luft and Olivecrona, 1953; Pearson et al., 1956; Pearson and Ray, 1959b).

Paradoxically, it was discovered that while physiological amounts of hormone could stimulate tumor growth (Crile, 1958; Huseby, 1965; Clifton and Sridharan, 1975; Kelly et al., 1979), the administration of pharmacological doses of hormones, such as estrogens (Haddow et al., 1944) or androgens (Nathanson, 1952) could induce tumor regression. More recently, a class of compounds known as antiestrogens have also been shown to inhibit estrogen-induced tumor growth in rat uterus and proliferation of hormone-dependent neoplasms. (Huggins, 1967; Cole et al., 1971; Ward, 1973; Jorden et al., 1976a, 1976b).

The outcome of early clinical trials of treating advanced breast disease with synthetic estrogens firmly established hormonal therapy as a valuable mode of treatment. However, only 25-30% of human breast cancers were

considered to be hormone-dependent and thus responsive to endocrine manipulation (McGuire et al., 1975b; Jensen, 1981). The problem facing clinicians treating breast cancer was distinguishing those patients who would respond to endocrine treatment, from those who could best be managed by chemotherapy. The development of estrogen receptor assays provided a useful tool for separating these two groups.

b) Steroid Action within the Cell

Steroid hormones exert their biological effect upon target tissues by binding to appropriate steroid receptor proteins within the cell. Human breast carcinoma tissue has been shown to contain various steroid receptors and measurements of the estrogen and progesterone receptor content of human breast tumors has enabled the clinician to more accurately identify those patients who would benefit from endocrine manipulation (McGuire and Chamness, 1973; McGuire et al., 1975b).

The first indication that physiological responses of target tissues were brought about by steroid interaction with specific macromolecular proteins (receptors) was demonstrated when Glascock and Hoekstra (1959) and Jensen and Jacobson (1962), using tritiated estrogens, observed that estrogens could be selectively concentrated and retained by estrogen-responsive tissues of various animals.

The receptor, also called estrophilin, was confirmed to be a protein by various analyses, such as stereospecificity and gel filtration chromatography (Walters, 1985). Receptors are limited in number, have high affinity for a specific ligand, and are concentrated in target tissues. The criteria used to identify steroid receptor proteins are described in detail by Clark and Peck (1981). Receptor levels are highest in tissues of the reproductive system (Toft and Gorski, 1966; Jensen and DeSombre, 1972; O'Malley and Means, 1974; Gorski and Gannon, 1976) and are also found to a lesser extent in pituitary, hypothalamus and various other organs (Noteboom and Gorski, 1965).

Much of the current understanding of the mechanism of steroid-induced biological response has been provided by observing the physiological changes in animal target tissues. Although biological responses have been extensively analysed in some hormone systems, for example, estrogenic stimulation of uterine growth (see review by Walters, 1985), the exact mode of steroid action remains undetermined.

The first model (Figure 1) to describe steroid-receptor interaction was called the two-step model (Shyamala and Gorski, 1967; Gorski et al., 1968; Jensen et al.,1968). This model has been referred to as the two-step model because it was thought that the interaction of steroid with cytoplasmic receptor first converted the receptor into an activated form (transformation) which was then capable of

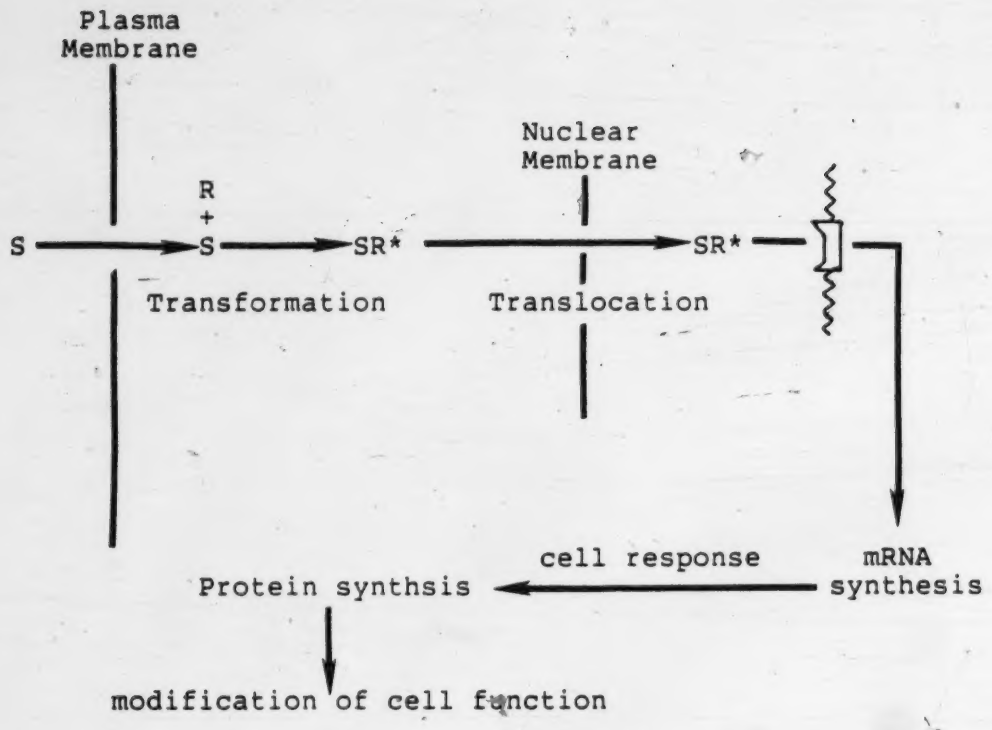

Figure 1. Two-step model of steroid-receptor interaction and induction of cellular responses.

a) Lipophilic steroid hormones (S) diffuse freely through the plasma membrane and bind to cytoplasmic receptors.

b) Steroid binding to the receptor results in a transformation of the receptor (SR*).

c) Transformation of the receptor allows the SR* to penetrate the nuclear membrane.

d) After translocation, the SR* interacts with chromatin to regulate cellular function.

penetrating the nucleus (translocation). Steroid-receptor complexes then become associated with chromatin in an interaction that stimulates nuclear RNA polymerase activity to induce biological responses as defined by synthesis of various proteins (Mueller et al., 1958, Palmiter, 1972; Schimke et al., 1975; Yamamoto and Alberts, 1976; MacKnight and Palmiter, 1979; Tata and Smith, 1979).

There is a lack of uniformity in the terminology used to describe the individual reactions associated with specific changes in the physical properties of the estrogen receptor. Throughout this paper, "transformation" refers to the reaction by which the 8S or 4S estrogen-receptor complex is modified to the 5S dimer and is altered from a state with fast to slow dissociation rates (Bailly et al., 1980; Milgrom, 1980). The term "activation" is used to describe those changes in the estrogen-receptor complex which lead to increased binding of the receptor to nuclei or DNA (Bailly et al., 1980; Milgrom, 1980; Walters, 1985).

Recently, the classical two-step model has been challenged by the affinity model (Figure 2) proposed by Walters (1985). The affinity model, which represents a composite of theories based on the observations of several investigators, depicts receptors in dynamic equilibrium between cytoplasm and nucleus. The distribution, predominantly nuclear, is determined by receptor concentration and cell volume. The affinity of receptors for nuclear components depends upon individual molecular

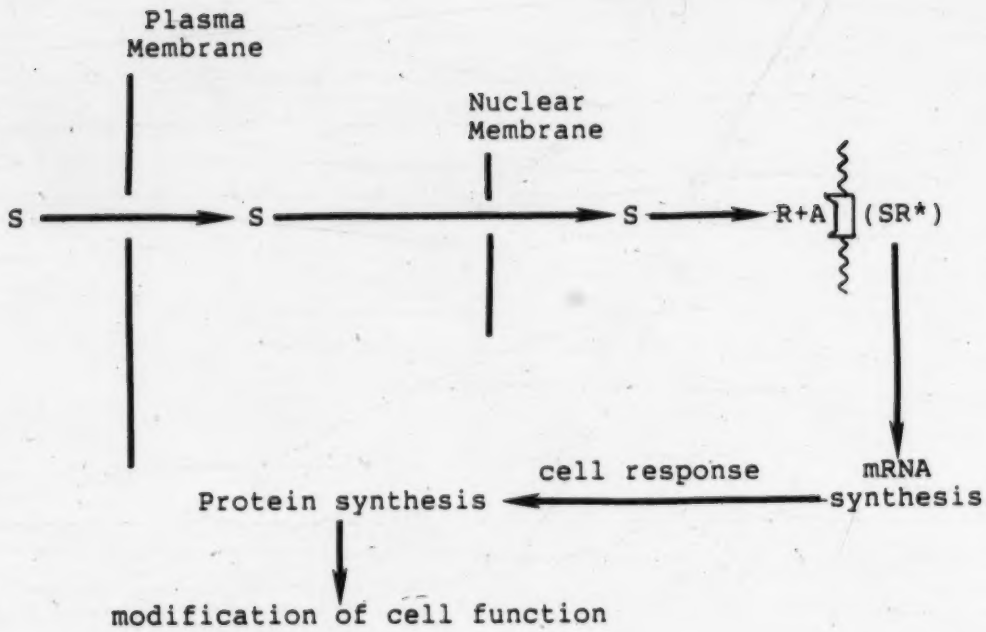

Figure 2. Simplified schematic of the Affinity Model of steroid-receptor interaction and induction of cellular responses (Walters, 1985).

a) Lipophilic steroid hormones (S) are distributed within both cytoplasmic and nuclear compartments.

b) Unoccupied receptors (R) are predominantly nuclear in an equilibrium binding state with chromatin components.

c) Steroid binding to receptors results in transformation of receptors into a biologically active form (SR*).

d) The affinity (Ka) of the transformed receptor for the nuclear acceptor sites (A) is increased, resulting in induction of mRNA polymerase activity, protein synthesis, and regulation of cell function (Walters, 1985).

properties of the various receptor proteins. As with the classical two-step model above, the affinity of the receptor for DNA increases when the receptor is bound by steroid (Skafar and Notides, 1985). Although this model describes most receptor systems, there is no completely satisfactory model that can be applied to all systems and as Walters points out, even her revised model will most likely continue to change as more highly sophisticated experimental techniques are developed.

The classical two-step model for steroid action was derived from early biochemical and autoradiographic studies of the intracellular localization of steroid receptors (Walters, 1985). Differential centrifugation indicated that, in target tissues not previously exposed to steroid, receptors were located in the cytosol (supernatant fraction obtained by high speed centrifugation), whereas in tissues exposed 'in vivo' or 'in vitro' to steroid, receptors accumulated in the nuclear fraction (Jensen and Jacobson, 1962; King et al., 1965; Gorski et al., 1968; Brecher et al., 1967 Jensen et al., 1968).

Sucrose gradient analysis of the estrogen-receptor binding reaction at 37°C demonstrated a depletion of unoccupied 8S cytosolic receptors with a concomitant increase in occupied 5S nuclear receptors (Gorski et al; 1968; Jensen and DeSombre, 1973). This phenomenon, which did not occur at 4°C, led to the conclusion that receptor transformation and nuclear localization were temperature-

dependent (Jensen et al., 1968; Vonderhaar et al., 1970; Giannopoulous and Gorski, 1971). The concept that cytoplasmic receptors migrated into the nucleus upon binding to steroid was further supported by early autoradiographs which showed obvious nuclear localization of [^{3}H]estradiol at 37^{o}C but not at 4^{o}C (Ullberg and Bengtsson, 1963; Stumpf, 1968a; 1968b; 1969; Jensen et al., 1969).

Over time, other models describing steroid-cell interaction were formulated and the theory of cytoplasmic localization and nuclear translocation of receptors was gradually modified (Williams and Gorski, 1972a; 1972b; DeSombre et al., 1975; Gannon et al., 1976; Gannon and Gorski 1976; Sheridan et al., 1979; 1981; Traish et al., 1981; Miyabe and Harrison 1983; Raaka and Samuels, 1983, Gorski et al., 1984; Callard and Mak, 1985). Several investigators studying different hormone systems, claimed that receptors existed within nuclei in the absence of steroid (Martin and Sheridan, 1882; Mester and Baulieu, 1972; 1975; Garola and McGuire, 1977a, 1977b; Zava et al., 1977a; 1977b; Linkie and Siiteri, 1978; Walters et al., 1978; 1980; Carlson and Gorski, 1980; Giannopoulous et al., 1980; Maeda et al., 1983; Rousseau, 1984), and experimental procedures of autoradiography (Sheridan 1975; Sheridan et al., 1979; cellular enucleation (Gorski and Raker, 1973; Veomett et al., 1974; Welshons et al., 1984), and immunocytochemistry (McClellan et al., 1984; Greene et al., 1984; King and Greene, 1984; Perrot-Applanat et al., 1985;

Miller et al., 1985; Molinari et al., 1985), supported these observations. The various theories describing steroid-hormone interaction with target cells were subsequently amalgamated by Walters (1985) into the affinity model, described above.

c) Nuclear Estrogen Receptor Binding and Biological Responses

In some systems such as chick oviduct, the relationship between receptor saturation by estrogen and synthesis of ovalbumin is exponential (Mulvihill and Palmiter, 1977; Walters, 1985). In some systems such as rat uterus, a simple linear relationship exists between the number of occupied estrogen receptors and the synthesis of a specific "induced protein" (IP) (Katzenellenbogen, 1980). However, the number of occupied nuclear estrogen receptors in rat uterus does not correlate well with the dose-response curve of early uterotrophic responses (Anderson et al., 1972a; 1972b; 1973; 1975; Stancel et al., 1973).

i) Nuclear receptors and uterine growth

Within 1-2 hours after exposure to pharmacological doses (1.0-2.5 µg) of estradiol, 100% of the nuclear estrogen receptors are occupied but early metabolic events do not result in true uterine growth (Clark and Peck, 1976). The true growth response of uterus occurs after 6 hours when the number of occupied nuclear estrogen receptors has

declined to 10-15% of the total amount. Clark and Peck (1976) hypothesized that if only 10-15% of nuclear receptors were required to initiate uterine growth, these receptor-nuclear acceptor sites must be different from the remaining 85-90% of sites observed 1 hour after rats were exposed to estradiol. Nuclei were then extracted with 0.4 M KCl to test for differential extractability of nuclear estrogen-receptor complexes. The results indicated that while most of the receptors could be extracted with 0.4 M KCl (KCl-soluble receptors), the number of receptors remaining in the nuclear fraction despite KCl extraction (KCl-insoluble receptors) corresponded to the number of sites estimated to be required for true uterine growth. Thus, Clark and Peck suggested that KCl-insoluble receptors were responsible for estrogen-mediated biological effects.

ii) KCl-insoluble receptors

Although KCl-insoluble receptors have been reported for other receptor systems (Puca and Bresciani 1968, Bruchovsky and Wilson, 1968; Best-Belpomme et al., 1975), the physiological significance of these receptors has not been established and their existence has been questioned. Assay conditions (Barrack et al., 1977, 1979) as well as methods of extraction (Juliano and Stancel, 1976; Muller et al., 1977, Traish et al., 1977), are known to influence the amount of receptor that can be extracted and consequently the amount that appears to be KCl-insoluble.

d) Nuclear Localization of Receptors

Although it is now generally accepted that steroid receptors are localized within the nuclear compartment of target tissues and of hormone-dependent breast tumors (Walters, 1985), the precise nuclear component remains undefined. Barrack and Coffey (1980) proposed that KCl-insoluble steroid-receptor complexes (Barrack et al., 1977) were associated with the nuclear matrix, a nucleic acid-depleted framework of nuclear nonhistone proteins. The nuclear matrix is thought by some investigators to play an important role in DNA replication and hnRNA synthesis (Berezeny and Coffey, 1976; Agutter and Birchall, 1979; Barrack and Coffey, 1982; Pietras and Szego, 1984).

Recent studies using purified chromatin rather than the nuclear matrix suggest that steroid receptors bind to nuclear acceptor sites which are composed of a DNA-protein complex of chromatin-associated nonhistone proteins and the DNA backbone (Steggles et al., 1971; Spelsberg et al., 1983; 1984). These findings suggest that nuclear matrix binding by steroid receptors observed by some groups, may also be affected by extraction procedures.

In summary, it appears that steroid receptors are associated with specific acceptor sites located on the chromatin and when steroids interact with receptors, the receptors are altered such that their effect is de-repressed. Consequently, RNA polymerase activity, and thus

physiological events, can be initiated (Mohla et al., 1972; Silva et al., 1977; Singh et al., 1986).

3. Historical Development of Estrogen Receptor Assays for Human Breast Carcinoma

Shortly after reports of specific estrogen-binding components in estrogen-responsive reproductive tissues of laboratory animals appeared (Glascock and Hoekstra, 1959), Folca et al. (1961) demonstrated that the uptake of labelled hexestrol 'in vivo' by breast tumors was higher in patients who responded to adrenalectomy than in those patients who did not respond. But, because most of the radioactivity was excreted in the urine, this method was not considered to be satisfactory for the characterization of breast tumors.

After Mobbs (1966) demonstrated specific [^{3}H]estradiol uptake by hormone-dependent rat mammary tumors, and Jensen et al. (1967) devised methods to study estrogen-receptor interaction 'in vitro', clinical trials were begun to determine if the response of breast cancer patients to endocrine ablation was correlated with specific binding of estradiol by their tumor slices.

With the formulation of the two-step mechanism of hormone action (Gorski et al., 1968; Jensen et al., 1968), Jensen and colleagues (1971) speculated that if some breast carcinoma cells depended on estrogenic stimulation for growth, hormone-dependent breast carcinoma cells might be

expected to contain the cytoplasmic estrogen receptor, whereas, if the tumor had lost its hormonal dependency, it would no longer produce the receptor. Thus, clinical responses were correlated with the presence of estrogen receptor in the cytosolic fraction of tumor homogenates (Jensen et al., 1971). The results indicated that breast cancers with low estradiol-binding or lacking cytosolic estrogen receptor rarely responded to endocrine treatment, whereas most patients whose tumors contained significant amounts of receptor, received objective benefit from hormonal therapy. These findings were confirmed and extended by reports from other investigators (Wittliff et al., 1972; Wittliff, 1974; Leclercq et al., 1973; 1975; Engelsman et al., 1973; Leung et al., 1973; Maass, 1972; Maass et al., 1975; McGuire et al., 1974; Savlov et al., 1974), and the observations from several laboratories were presented at a workshop sponsored by the Breast Cancer Task Force of the National Cancer Institute (McGuire et al., 1975b).

The Task Force concluded that despite the variety of analytical methods used for determining receptors, breast tumors lacking estrogen receptor rarely responded to endocrine therapy. Most primary and metastatic breast cancers were found to contain some detectable receptor, but 60-70% could be classified as 'receptor-poor' and were unresponsive to hormonal treatment. Although the percentage of breast tumors reported to be 'receptor-rich' later increased to 70-85% (McGuire et al., 1975b), one third

of the tumors that contained estrogen receptors also failed to respond to hormonal therapy.

The rise in the incidence of the detection of receptor-positive tumors has been attributed to increased care in the handling of tissue samples as well as to improved assay techniques which minimize receptor degradation (McGuire et al., 1975a).

4. Significance of Progesterone Receptors and Nuclear Estrogen Receptors

Since only 54% of women with breast tumors containing estradiol-binding sites responded to therapy (Proceedings of the NIH Consensus Meeting, 1980) it was obvious that a more accurate indicator of hormone-dependent tumors was required. As a result, two approaches have been generally taken for assessing the integrity of the estrogen-receptor mechanism in human breast tumors.

One procedure depends on the simultaneous quantification of both estrogen and progesterone receptor levels (Horwitz et al., 1975; McGuire and Horwitz, 1977). This approach was based on the hypothesis that the biosynthesis of progesterone receptor is controlled by estrogen action (Toft and O'Malley, 1972). Therefore, breast tumors containing both receptors would be expected to retain hormonal responsiveness (Horwitz and McGuire, 1977; 1978; 1979). Indeed, the clinical response for patients with

tumors containing both cytosolic estrogen and progesterone receptors was found to be approximately 74% (Edwards et al., 1979; Osborne et al., 1980). Consequently, assays to determine the presence of both estrogen and progesterone receptors in human breast cancers are currently performed by major laboratories on a routine basis.

Another method has been to determine both the cytosolic and nuclear estrogen receptor content of breast tumors (Laing et al., 1977; Cowan et al., 1984). This approach was based on the hypothesis that the absence of nuclear estrogen receptor in the presence of cytosolic estrogen receptor would indicate a defect in the translocation process (Thorsen and Stoa, 1979). Initial studies correlating the presence of nuclear estrogen receptors in human breast tumors with patient response indicated that approximately 71% of tumors containing both soluble and nuclear estrogen receptors underwent objective regression as opposed to only 54% of tumors with only cytosolic estrogen receptors (Leake et al., 1981a; 1981b). Several other groups have since studied nuclear estrogen receptor content in relation to both patient management and prognosis (Garola and McGuire, 1977a, 1977b; Barnes et al., 1979; Bishop et al., 1979; Fazekas and MacFarlane, 1980; Hahnel et al., 1980; O'Connell et al., 1982) and the results of these studies are comparable to the clinical response observed for patients whose tumors contain both cytosolic estrogen and progesterone receptors.

5. Limitation of Receptor Assays

The assessment of concentrations of estrogen and progesterone receptors in human mammary tumor specimens has been significant for the selection of therapy in the management of breast carcinoma (McGuire et al., 1975b; 1978; 1982; Wittliff et al., 1976). However, approximately 25% of patients whose breast tumors contain estrogen and progesterone receptors fail to respond to endocrine treatment. Also, there is a 5-10% response rate to endocrine therapy in patients with estrogen receptor-negative tumors (Lippman, 1980; Seibert and Lippman, 1982).

The failure of some breast tumors to respond to treatment may be explained by the fact that tumors have a high degree of cellular heterogeneity and these various tissue types are known to contain mixed populations of both receptor-positive and receptor-negative cells (Allegra et al., 1979; Wittliff, 1981; Woodruff, 1983). A receptor-positive assay would have little prognostic value if the tumor contained a large number of receptor-negative cells. Also, tumors are often associated with significant amounts of stromal material which usually requires high shearing forces to fragment the tissue and to disrupt the plasma membrane (Dounce, 1963). Excessive and potentially damaging homogenization can be avoided by grinding the tissue to a fine powder in liquid nitrogen (Wittliff et al., 1980). This

procedure also provides a uniform distribution of cell types and thus minimizes inaccurate receptor determinations due to heterogeneity.

A tumor response to endocrine therapy, indicated by tumor regression, may occur in some tumors which apparently lack estrogen receptors. Degradation or inactivation of some receptors may occur due to improper storage or excessive handling of the tumor specimen. Also, some receptors which may be occupied by endogenous steroids may not be quantified due to inappropriate assay conditions. Endogenous estrogens or therapeutic antiestrogens occupying nuclear estradiol-binding sites do not appear to undergo significant exchange with [^{3}H]estradiol at low temperatures used for routine receptor assays (Edwards et al., 1980). This may explain the lower incidence of cytosolic estrogen receptor content observed in breast tumors from premenopausal patients (20% versus 64% of breast tumors from postmenopausal patients, Hahnel, 1981).

The two approaches mentioned above for assessing estrogen-receptor interaction both provide a more accurate correlation between receptor status, hormonal senstivity, and clinical responsiveness than the method based on the quantification of cytosolic estrogen receptors alone. Both of these approaches were taken in an attempt to identify the various types of receptor defects that are now known to disrupt the biological responsivess of some cells to a steroid stimulus (Wittliff, 1984), yet interestingly, the

relationship between cytosolic and nuclear estrogen receptors and progesterone receptors has not been studied in detail (Romic-Stojkovic and Gamulin, 1980).

Additionally, the full potential of nuclear estrogen receptor assays has not yet been completely explored. Nuclear estrogen receptor concentrations of human breast tumors have been determined mainly by KCl-extraction of nuclei, a procedure which may miss KCl-insoluble receptors which may be of biological importance.

6. Summary

The development of estrogen receptor assays has provided valuable information for the prognosis and selection of therapy for patients with breast cancer. The presence of cytosolic estrogen receptor in the tumor has been related to increased disease-free interval and total survival time (Knight et al., 1977; Osborne and McGuire, 1979; Hawkins et al., 1980; McGuire et al., 1982); the incorporation of either progesterone receptor or KCl-soluble nuclear estrogen receptor data has improved the accuracy of identifying those patients who may have a better prognosis. However, the inclusion of both cytosolic progesterone receptor and total nuclear estrogen receptor data in routine assays may further improve the accuracy of identifying those patients who may benefit from endocrine therapy.

7. Research Objective

The purpose of this research was to compare the data for total nuclear estrogen receptor content of human breast tumors with the data for cytosolic estrogen and progesterone receptors. In order to accomplish this it was necessary to:

i) validate the separation techniques required to isolate [^{3}H]estradiol-receptor complexes from free and non-specifically bound [^{3}H]estradiol.

ii) evaluate the reliability of the nuclear estrogen receptor assay and also establish a reference tissue for this assay.

iii) apply the nuclear assay to test both KCl-soluble and KCl-insoluble fractions of nuclei for the presence of estrogen receptors in a small series of breast tumor samples.

CHAPTER II

VALIDATION OF BINDING ASSAY TECHNIQUES

1. INTRODUCTION

The first research objective was to validate assay techniques required for the isolation of estrogen-receptor complexes. Assay methods for the determination of estrogen (and also progesterone) receptor concentrations of target tissues are generally based on the amount of tritiated steroid that binds to receptor proteins present in the cytosol fraction of tissue homogenates (Chamness and McGuire, 1979). [^{3}H]estradiol binds not only to receptor proteins but also to plasma proteins such as albumin, glycoprotein, and sex-hormone binding globulin (SHBG) (Chamness and McGuire, 1979), which often contaminate human tumors (Maass et al., 1975).

Increasing concentrations of [^{3}H]steroid and a parallel range of unlabelled steroid competitor are used to determine specific steroid binding sites under saturation conditions. Diethylstilbestrol (DES) is usually used as a nonradioactive, competitive ligand in the presence of [^{3}H]estradiol in order to estimate radioactivity that binds to proteins other than receptors. DES equals or exceeds estradiol in its affinity for receptors, but has a very low affinity for plasma proteins such as SHBG. Therefore, DES occupies most of the receptors, yet has no effect on estradiol that binds to nonreceptor proteins (Chamness and McGuire, 1979).

Current routine assays for detection of estrogen receptor levels differ mainly in the procedures used to separate [^{3}H]steroid bound to receptor, from free or nonspecifically bound steroid (Godefroi and Brooks; 1973; Gore-Langton et al., 1973; Chamness and McGuire, 1979). Adsorption procedures are generally fast and easy to use. Other techniques such as equilibrium dialysis are not as well studied and are lengthy and difficult to quantitate. Despite the availability of several assay techniques, there is no single method that can be used to isolate [^{3}H]-estradiol-receptor complexes quickly and efficiently from both the cytosolic and nuclear fractions of target tissues.

The dextran-coated charcoal (DCC) assay is the most widely used method to isolate those receptors which can be solubilized into the cytosol fraction (Feherty et al., 1971; Hawkins et al., 1975; McGuire and Chamness, 1973), but other techniques must be employed to isolate receptors which are intricately associated with the nuclear fraction. The dextran-coated charcoal assay is popular because it is rapid, simple, accurate and economical (Seibert and Lippman, 1982). Exposure of cytosol to dextran-coated charcoal followed by centrifugation removes free [^{3}H]-steroid, while leaving receptor-bound [^{3}H]steroid in solution. Charcoal has a moderate affinity for steroids and thus can minimize low-affinity binding to cytosol proteins. The inclusion of dextran and BSA in the charcoal suspension reduces adsorption of estrogen-receptor complex to the

charcoal (Korenman, 1968; Chamness and McGuire, 1979; Powell et al., 1981). However, dissociation of [^{3}H]steroid from the receptor complex may occur if exposure is prolonged, temperatures are elevated (Chamness and McGuire, 1979), or buffers of high ionic strength are used (Peck and Clark, 1977). Moreover, under the conditions of the DCC assay, the receptor is susceptible to degradation by proteolytic enzymes which are present in nuclear extracts prepared from human breast tumors (Garola and McGuire, 1977a; 1977b). Therefore this method cannot be used to separate nuclear estrogen-receptor complex and is best suited to the isolation of unoccupied receptors which can easily be extracted into the cytosolic fraction.

In order to determine nuclear estrogen receptor levels, assay techniques that include extraction of receptors from isolated nuclei, and the use of higher incubation temperatures (30-37^{o}C) to exchange previously bound endogenous estrogen with [^{3}H]estradiol, are often used (Anderson et al., 1972a). In addition, assays for nuclear receptor must provide protection against receptor degradation from proteolytic enzymes which are present in the nuclear fraction (Edwards et al., 1980; Horwitz and McGuire, 1980). In the following study, two assays, Sephadex LH-20 and hydroxylapatite (HAP), were evaluated for their ability to separate [^{3}H]estradiol-receptor complexes from free and nonspecifically bound [^{3}H]steroid.

a) Sephadex LH-20 Chromatography

The technique of gel filtration chromatography using hydroxylated Sephadex LH-20 has been used by several groups to measure estrogen receptor present in the KCl extracts of purified nuclei of human breast cancer specimens (Godefroi and Brooks, 1978; Singhakowinta et al., 1975, 1976; Thorsen and Stoa, 1979; Martin and Sheridan, 1982; Vandewalle et al., 1983). This procedure takes advantage of differences in molecular size between free steroid and receptor-bound steroid.

Sephadex LH-20 is lipophilic and thus has high affinity for free steroid and retains it on the gel without appearing to affect the radioactivity that is specifically bound to receptor (Ginsberg et al., 1974). In addition, this method is a simple, accurate procedure which has the advantage of enabling one to process large numbers of samples with good estimates of Kd and number of binding sites. However, the nuclear pellet which remains following exposure to 0.4 M KCl is too thick and fibrous to pass through the gel, thereby rendering this method unsuitable for the separation of estradiol-receptor complexes that may remain in the salt-insoluble nuclear fraction.

b) Hydroxylapatite

The hydroxylapatite (HAP) assay has also been used by various investigators to effectively separate estrogen-receptor complexes which may be present in the nuclear fraction of tissue homogenates (Williams and Gorski, 1972a; 1972b; Garola and McGuire, 1977a; 1977b). Small molecular weight molecules do not associate readily with HAP while proteins bind rather tightly (Erdos et al., 1970). The steroid-receptor complex is adsorbed onto HAP while most of the plasma contaminants and free steroid are removed by washing the HAP several times with low ionic strength phosphate buffer. The HAP is then extracted with 95% ethanol to determine bound steroid (Erdos et al., 1970; Chamness and McGuire, 1979). Extensive washing at 4°C of HAP-bound estrogen-receptor complexes makes this a tedious procedure, inefficient for separating [^{3}H]steroid-receptor complexes from free and nonspecifically bound [^{3}H]steroid in a large number of samples.

Validation of assay techniques included comparing results obtained by the Sephadex LH-20 and HAP assays with results obtained by the DCC assay. The data were generated using cytosols prepared from uteri of untreated, immature female rats. Rat uterus was used as a source of tissue because previous studies using rat uterus (see review by Walters, 1985) have provided considerable information regarding both receptor content and steroid-cell interaction for this tissue. In addition, the animals are easy to

maintain, relatively inexpensive and readily available.

The receptor content of a target tissue can be determined from the data obtained from binding assays, by a variety of mathematical transformations. Scatchard analysis (Scatchard, 1949) of binding data (Table 1) is most often used (Chamness and McGuire, 1979) to calculate receptor content, as well as to obtain the dissociation constant (Kd) for steroid binding to receptor. Scatchard analysis of steroid-binding data is simply a graph in which the concentration of bound/free steroid (vertical axis, Y) is plotted against the amount of specifically bound steroid (horizontal axis, X) for each concentration of steroid used (Figure 3). The best straight line is drawn through these points connecting both axes. The total number of steroid-receptor complexes is calculated from the X-intercept (n). The dissociation constant, a measure of the affinity of steroid for receptor, is calculated from the negative reciprocal of the slope of the line. The amount of receptor protein is generally expressed as femtomoles (10^{-15}M) per uterus (Clark and Peck, 1976), or as femtomoles per mg of cytosol protein (Chamness and McGuire, 1979). The concentration of receptor may also be expressed as fmol per g of tissue (O'Connell et al., 1982) or as fmol per mg of DNA (Wittliff, 1984). These latter two modes of expression are particularly useful for nuclear steroid receptors.

Table 1

Sample calculations of binding data for Scatchard analysis of the estrogen receptor content of rat uterine cytosol.

Specific activity, [^{3}H]-estradiol: 91 Ci/mmol
= 202 DPM/fmol

Uterine weight = 0.25 mg/uterus

[^{3}H]-E	Total Counts	Total Bound	NSB	TB-NSB	Y	Specific Bound (B)
nM	DPM	DPM	DPM	DPM	B/F	fmol/ml
5	253465	11914	1906	10008	0.041	200.2
2	104615	10660	765	9895	0.105	197.9
1	51850	9266	381	8885	0.209	177.7
0.5	25095	8228	180	8048	0.477	161.0
0.2	10650	5285	172	5113	0.953	102.3
0.1	5710	3366	35	3331	1.420	66.6

For the Scatchard plot (Scatchard, 1949), specifically bound steroid (B) is plotted (x-axis) against Bound Steroid/Free Steroid (B/F) as shown in Figure 3. TB = Total Bound
NSB = Non Specifically Bound

$$\text{Binding capacity} = \frac{\text{x-intercept (fmol/ml)}}{\frac{\text{Cytosol vol (ml)}}{\text{Reaction vol (ml)}} \times \text{Uterine Weight}} = \frac{\text{fmol bound}}{\text{uterus}}$$

$$= 1020 \frac{\text{fmol}}{\text{uterus}}$$

$$\text{Dissociation constant (Kd)} = -\frac{1}{\text{slope}} = 0.100 \text{ nM}$$

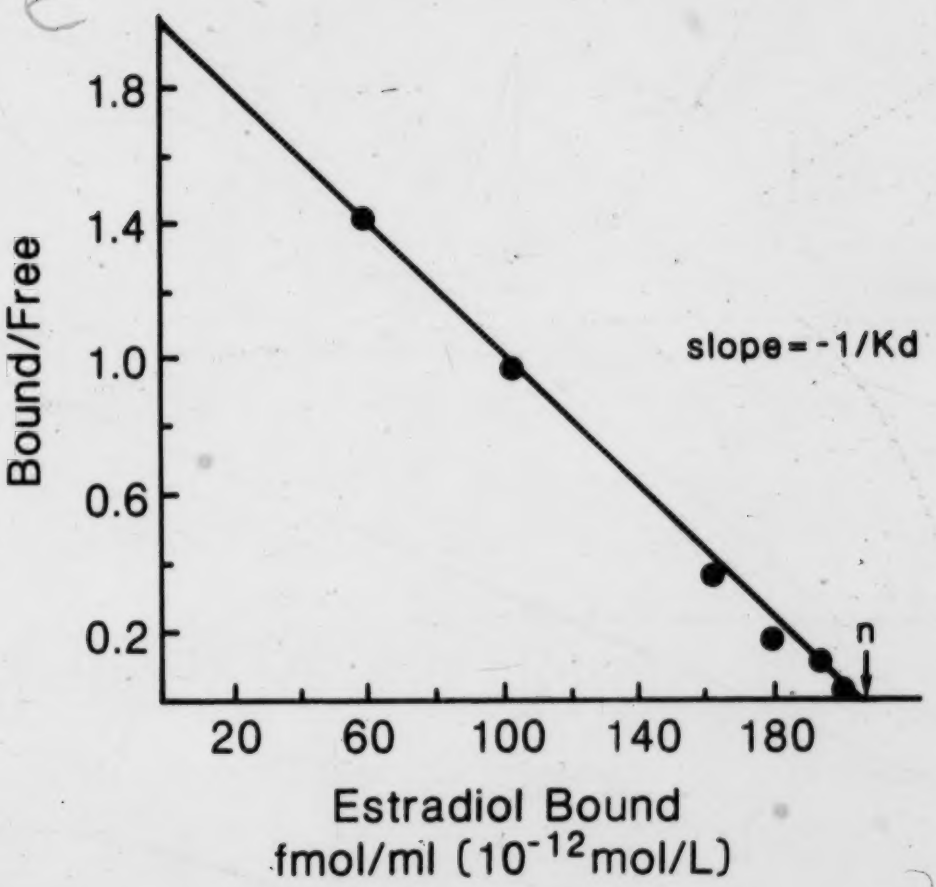

Figure 3. Typical Scatchard plot.

2. MATERIALS and METHODS

a) Chemicals

[2,4,6,7-^{3}H]estradiol, specific activity 92 Ci/mmol, was purchased from New England Nuclear (Boston, MA, USA). Dextran, bovine serum albumin (BSA), diethylstilbestrol (DES), calf thymus DNA, dithiothreitol (DTT), monothioglycerol (MTG), activated charcoal and ethylenediaminetetraacetic acid (EDTA), were obtained from Sigma Chemical Co. (St. Louis, MO., USA). Tris-HCl was obtained from Shwartz/Mann (Spring Valley, NY, USA). Hydroxylapatite (HAP) was obtained from Bio-Rad Laboratories (Richmond, CA, USA), Sephadex LH-20 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Ready-Solv HP scintillation fluid was obtained from Beckman Instruments (Fullerton, CA, USA). Ethanol (95%) was obtained from Stanchem (Winnipeg, Man). All other chemicals were purchased from Fisher Scientific Co. (Quebec, Canada).

b) Buffers

1. Tris-MoO_4: 10 mM Tris-HCl, 1.5 mM EDTA, 10% glycerol (v/v), 10 mM sodium molybdate (Na_2MoO_4), 12 mM MTG, pH 7.4 at 22°C

2. TK.4: 10 mM Tris-HCl, 0.4 M KCl, pH 7.0 at 22°C

3. TDK.4: 10 mM Tris-HCl, 10 mM DTT, 0.4 M KCl, pH 7.0 at 22°C

4. TKP: 50 mM Tris-HCl, 10 mM KH_2PO_4, pH 7.2 at 22°C

5. KH_2PO_4: 10 mM potassium phosphate, pH 7.2 at 22°C

c) Suspensions:

1. DCC: 10 g/L charcoal (Norit A), 1.0 g/L Dextran 500, 1.0 g/L BSA in Tris-MoO_4 buffer

2. LH-20: a 1:1 ratio of hydrated Sephadex LH-20 beads in TK.4 buffer (10 mM Tris-HCl, 0.4 M KCl, pH 7.0 at 22°C)

3. HAP: 100 g/L in TKP buffer (50 mM Tris-HCl, 10 mM KH_2PO_4, pH 7.2 at 22°C

Double-distilled water was used to prepare buffers.

d) Animals

Female Sprague-Dawley rats, 5-6 weeks old, were obtained from the Biosciences Animal Services at the University of Alberta. Rats were killed by carbon dioxide asphyxiation; the uteri were excised, cleaned of adhering fat and mesentery, rinsed in cold 10 mM KH_2PO_4 buffer and blotted. Uteri were ground to a fine powder in liquid nitrogen and stored at -70°C until required.

e) Preparation of the Cytosol

The cytosol was prepared and assayed as outlined in Figure 4. All procedures were carried out at 0-4 °C. Approximately 0.2-0.5 g of powdered rat uteri that had been

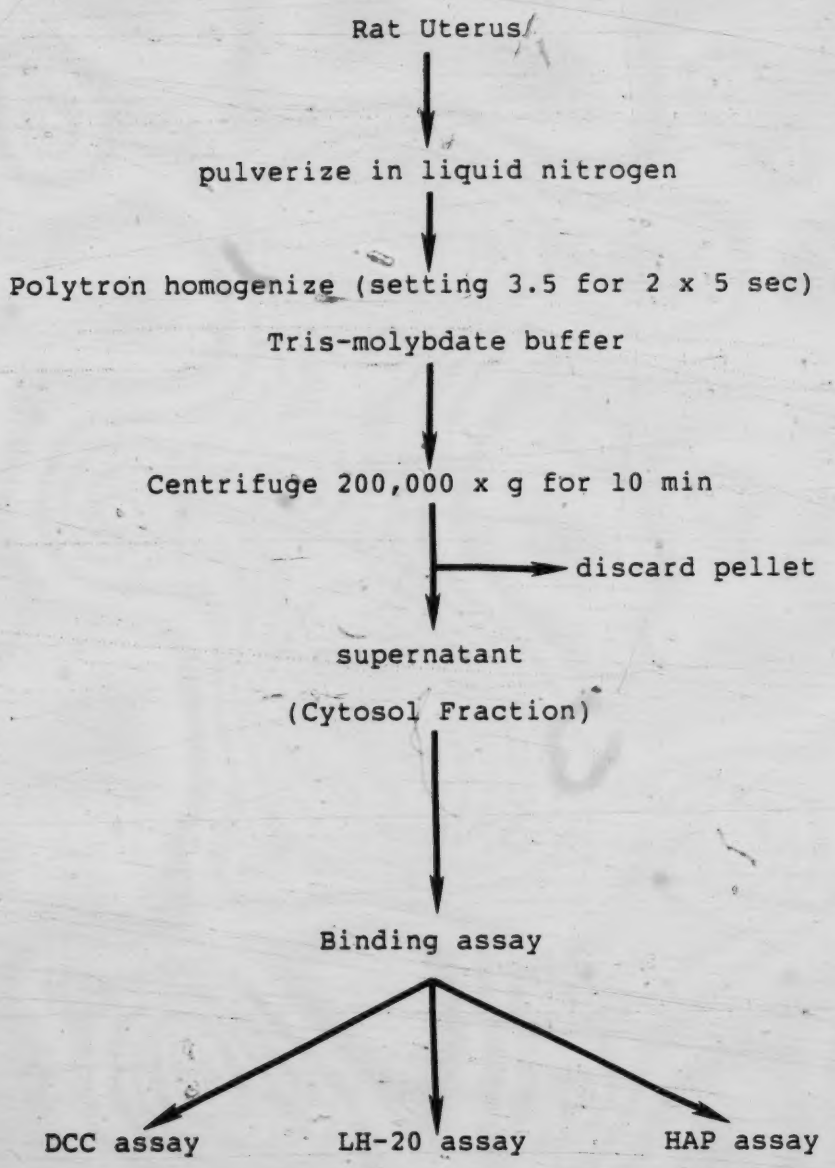

Figure 4. Flow diagram for the preparation and assay of the cytosol fraction.

stored at -70°C were homogenized on ice in Tris-MoO_4 buffer using a Polytron PT-10 homogenizer (Brinkmann) set at 3.5 for two 5-second bursts. The homogenate was centrifuged in a Beckman L8-70M ultracentrifuge at 200,000 x g for 30 min in a SW60 Ti rotor. The resulting supernatant (cytosol fraction) was decanted and diluted to approximately 1.0-2.0 mg of protein/ml of Tris-MoO_4 buffer. The protein concentrations were determined by the method of Lowry et al. (1951).

f) Binding Assays

Aliquots of rat uterine cytosol (200 µl) were incubated overnight at 4°C with 50 µl of one of 6 concentrations (0.1-5.0 nM) of [^{3}H]estradiol. Following an overnight incubation, the experimental samples were exposed to DCC, LH-20 and HAP as described below. The amount of tritiated estradiol that was bound to non-receptor proteins was determined by linear regression from a series of three tubes containing a 100-fold excess of DES and was subtracted from the total amount of bound radioactivity to yield a measure of estradiol specifically bound to receptor. The total number of specific estradiol-binding sites and the dissociation constant were determined by analysis of Scatchard plots (Table 1 and Figure 3, Scatchard, 1949). For the purposes of this study, the total number of receptors was expressed as fmol/uterus. The uterine weights used represented the mean weight obtained from twelve uteri.

i) Dextran-coated Charcoal Method

A 500 µl aliquot of dextran-coated charcoal (DCC) suspension was added to the cytosol and incubated at 0-4°C for 10 min. The charcoal and adsorbed free [^{3}H]estradiol were precipitated by centrifugation at 12,800 x g in an MSE Micro Centaur microcentrifuge. A 500 µl aliquot of the resulting supernatant was mixed with 5.0 ml of Beckman scintillation counting fluid and the radioactivity was measured using a Beckman LS9000 liquid scintillation counter.

ii) Sephadex LH-20 Method

Sephadex LH-20 was prepared by hydrating the beads for 3 hrs at room temperature in TK.4 buffer. The gel was washed twice with TK.4 buffer and resuspended in TK.4 buffer to give a 1:1 ratio of gel to buffer. The LH-20 had been swollen for less than one week before use. Columns were prepared by placing a 3 mm glass bead in the bottom of a disposable, blue plastic, 1.5 ml pipette tip and filling the tip with approximately 1.2 ml of prepared gel. The columns were washed once with 400 µl of TDK.4 buffer; 100 µl of sample were applied to columns at 4°C, washed with 100 µl of TDK.4 buffer and eluted with 400 µl of TDK.4 buffer directly into scintillation vials; 5 ml of scintillation fluid were added to each vial and the radioactivity was determined as above.

iii) Hydroxylapatite Method

Bio-Gel HTP hydroxylapatite (HAP) was prepared by suspending 100 g/L of HAP in TKP buffer and washing the HAP 3 times in the same buffer. The settled HAP was resuspended in TKP buffer in a ratio of one volume of HAP to five volumes of TKP buffer and 500 µl were added to each tube containing 500 µl of TKP buffer and 200 µl of cytosol. The mixture was incubated for 30 min at 4°C, stirring every 10 min on a vortex mixer, followed by centrifugation at 12,000 x g for 2 min. The supernatant was decanted and discarded. The pellet was resuspended in 400 µl TKP buffer plus 100 µl of labelled steroid.

Alternatively, cytosol which had already been incubated with steroid as above as for the DCC assay, was then bound to HAP. The HAP was then washed and processed to radioactive counting as above.

Free steroid was removed by centrifugation at 12,800 x g for 2 min and washing the pellets 3 times with 1 ml of 10 mM KH_2PO_4 buffer; bound steroid was extracted by the addition of 500 µl of 95% ethanol to each tube. Both the ethanol extract and incubation tube were placed in a counting vial with 5.0 ml of scintillation fluid for determination of radioactivity.

3. RESULTS

To validate the binding assays, a series of rat uterine cytosols were prepared as described in the Materials and Methods section, and assayed for estrogen receptor using the techniques of DCC, LH-20 and HAP to separate estradiol-receptor complex from free and nonspecifically bound estradiol. Analysis of variance (α= 0.05, F = 1.00) of the results, as shown in Table 2, indicated that these separation techniques had no significant effect on the estimated receptor concentration. The estrogen receptor concentration as determined by the standard DCC assay was 954±141 fmol/uterus or 318±49 fmol/mg cytosol protein.

Initially, when the cytosol was incubated with HAP prior to the addition of steroid, the total number of receptor sites and dissociation constant appeared to be slightly lower than expected (results not shown), possibly due to some inaccessibility of the receptor binding site once it had adsorbed onto the HAP. By modifying the procedure slightly, (addition of 100 µl of steroid and 400 µl of buffer), data closer to the established mean were obtained (Table 1, HAP-2).

Table 2.

The concentration of estradiol-binding sites in the cytosol fraction of uteri of untreated immature rats. Binding data from Sephadex LH-20 and HAP assays were compared with binding data from the DCC assay.

		Concentration of Estradiol-Binding Sites	
		fmol/uterus	Dissociation Constant
Assay	(N)	(mean±S.D.)	(mean±S.D.; nM)
DCC	(16)	954±141 *	0.111±0.105
LH-20	(5)	1050±108	0.113±0.037
HAP-1	(6)	1038±144	0.113±0.130
HAP-2	(8)	1014±144	0.115±0.024

HAP-1 : HAP added after receptor was incubated with steroid.
HAP-2 : Receptor adsorbed onto HAP before steroid was added.

* Equivalent to 318 fmol/mg of cytosol protein.

Analysis of variance (α=0.05, F=1.00) showed no significant difference between the separation techniques of DCC, Sephadex LH-20 and HAP.

4) DISCUSSION

The concentration of estrogen receptor of cytosols prepared from uteri of untreated immature rats apparently can have a 2-3 fold range of values (243-551 fmol/mg of protein). These results were from assays performed on frozen cytosols prepared from two groups of rats and analysed over a six-month period by three personnel of the Hormone Receptor Laboratory of the Department of Medicine of the University of Alberta and Cross Cancer Institute, Edmonton. The mean receptor level for 89 consecutive cytosols was determined to be 373±70 fmol/mg of protein. The observed variation within batches of cytosol could be caused by technical inconsistencies in performing the assay or possibly by differential stability of aliquots of cytosol prepared and stored at -70°C (Love et al., 1983). Variations among batches of cytosol may be due to differences between animals (Shih and Lee, 1978). Older animals may have slightly higher levels of endogenous estrogen which could result not only in an increased affinity of the receptor for nuclear components but also in an increased rate of synthesis of the receptor protein (Kassis and Gorski, 1981; 1983). Or, the total cellular protein concentration and weight of the uteri may differ between younger and older rats. Other as yet unrecognized factors may also be involved.

The estrogen receptor concentration of rat uterine cytosols determined in this study was within the range of expected values (see above). The result of Sephadex LH-20 chromatography of receptor-bound radioactivity eluting in the void volume (400 µl) and in the same volume as blue dextran, was in agreement with results reported by Roy and McEwen (1977) and by Thorsen and Stoa (1979). Therefore, it was concluded that the separation techniques of Sephadex LH-20 and hydroxylapatite were functionaly equivalent to dextran-coated charcoal in their abilities to separate estrogen-receptor complexes from free and nonspecifically bound estradiol in cytosols prepared from rat uteri.

CHAPTER III

EVALUATION OF THE NUCLEAR ESTROGEN RECEPTOR ASSAY

1. INTRODUCTION

Having established the validity of techniques for the separation of bound from free steroid (Chapter II), the second research objective was to ensure that assay conditions for the determination of nuclear estrogen receptors provided accurate, reproducible results. The objectives of assays that are designed to measure specific nuclear proteins of target tissues are to isolate purified nuclei from cytoplasmic constituents and to protect the proteins from degradation while minimizing entrapment within the nuclear components (Mayer and Gulick, 1942; Zbarsky and Georgiev, 1959; Zbarsky et al., 1962; Widnell et al., 1969).

Tissues can be processed in a number of buffers using homogenizers such as the Polytron apparatus (Brinkmann). The homogenate can then be subjected to a variety of centrifugation procedures, which differ with regard to the speed and duration of centrifugation, depending upon the desired product (Steel and Busch, 1963; Busch and Daskal, 1977). The method most widely employed for the preparation of nuclei uses sucrose solutions for stabilization of nuclear structures (Busch and Daskal, 1977). Magnesium, usually coupled with potassium, is often used for ionic stabilization in nuclear isolation techniques because it is

required as a cofactor by a variety of nuclear-associated enzymes (Widnell and Tata, 1964). A buffering agent such as Tris or phosphate is required to maintain the pH of sucrose-magnesium solutions in the acidic range (pH 6-7), because under more alkaline conditions, divalent cations may become complexed with hydroxyl groups, causing distortion of nuclei and thereby poor nuclear preparations or low yields (Busch and Daskal, 1977).

Detergents are often included in procedures for isolation of nuclei, particularily from tumor tissues. Detergents such as Triton X-100 improve nuclear purity and yield by destroying cell membranes and by stripping off the outer nuclear membranes (Smuckler et al., 1976). Detergents also act to reduce tritiated estradiol binding to nonspecific proteins in human breast tumors (Syne et al., 1982).

Sulfhydryl reducing agents, such as dithiothreitol (DTT), are also included in solutions used to process nuclear fractions (Lieberburg and McEwen, 1979) in order to protect the receptor against against inactivation by oxidation of essential sulfhydryl groups (Seibert and Lippman, 1982). DTT has also been reported to inhibit ligand binding to low affinity, high capacity 'type II' estrogen binding sites in rat uterus (Markaverich et al., 1982), and to a lesser extent in human breast tumors (Syne et al., 1982; Panko and Clark, 1981).

In summary, steroid receptors are thermolabile, pH and ionic strength dependent, unstable proteins (Katzenellenbogen et al., 1973; Seibert and Lippman, 1982). Therefore, appropriate assay conditions that provide excellent nuclear yield while maintaining receptor integrity must be chosen in order to minimize underestimation of receptor content. In order to fulfill the second research objective, the nuclear estrogen receptor assay of Roy and McEwen (1977) was evaluated by quantifying the estrogen receptor content of uteri of immature rats. The extent of the assay variability was subsequently determined in order to use immature rat uteri as a reference tissue for the assay of nuclear estrogen receptors of human breast tumors.

Immature rats, presumably with no or low levels of endogenous estrogens, were estradiol-primed with a single pharmacological dose of estradiol in order to enhance the affinity of estrogen receptors for nuclear acceptor sites. The advantage of using rat uterus is that uterine nuclear estrogen receptor content is well documented (Walters, 1985) and it permits a reliable evaluation of assay procedures.

2. MATERIALS and METHODS

a) Chemicals

Sucrose was obtained from Schwartz/Mann (Spring Valley, NY, USA). Triton X-100 was purchased from BDH Chemicals (Toronto, Ontario). All other reagents were purchased from Fisher Scientific Co. (Quebec, Canada) or as described in Chapter II.

b) Buffers

1. NI: 1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, 0.25% (v/v) Triton X-100, pH 6.5 at 22°C

2. NII: 1 mM KH_2PO_4, 0.32 M sucrose, 3 mM $MgCl_2$, pH 6.8 at 22°C

3. NIII: 1 mM KH_2PO_4, 2.4 M sucrose, 1 mM $MgCl_2$, pH 7.0 at 22°C

4. TD: 10 mM Tris-HCl, 10 mM DTT, pH 7.4 at 22°C

5. TDK.4: 10 mM Tris-HCl, 10 mM DTT, 0.4 M KCl, pH 7.4 at 22°C

6. TDK.8: 10 mM Tris-HCl, 10 mM DTT, 0.8 M KCl, pH 7.4 at 22°C

7. Tris.MoO_4: 10 mM Tris-HCl, 1.5 mM EDTA, 10% glycerol, 10 mM sodium molybdate, 12 mM MTG, pH 7.4 at 22°C

Suspensions of Sephadex LH-20 and HAP used for nuclear estrogen receptor assays were described in Chapter II.

c) Animals

Female Sprague-Dawley rats, 5-6 weeks old, were estradiol-primed by intraperitoneal injection of 10 µg of 17β-estradiol in 200 µl of 5% ethanol in normal saline, 30 min prior to sacrifice. Control animals received no treatment before being sacrificed. Uteri were excised, stripped of adhering mesentery, pulverized, and stored at -70°C until required.

d) Preparation of the Nuclear Fraction

(i) Isolation of nuclei. The nuclear fraction was prepared and assayed for estrogen receptor as shown in Figure 5. Powdered uterine tissue was processed by the method of Roy and McEwen (1977). Tissues were homogenized as described previously, in 10 volumes of NI buffer and centrifuged in a Beckman J2-21 at 800 x g for 10 min at 4°C. The supernatant was discarded and the pellets were washed twice with 10 volumes of NII buffer. The pellet, comprised of nuclei and dense cellular components, was resuspended in NII buffer and diluted in 2.2 M sucrose (NIII buffer). The final, high-viscosity suspension was centrifuged at 10,000 x g for 1 h at 4°C, to sediment the nuclei, leaving most of the cytoplasmic constituents in suspension. The supernatant was decanted and discarded. The remaining sucrose was wiped from the sides of the centrifuge tube with a tissue. Sucrose-purified nuclei in pellets of selected samples were determined to be free of extensive cytoplasmic contamination by phase-contrast microscopy (not shown).

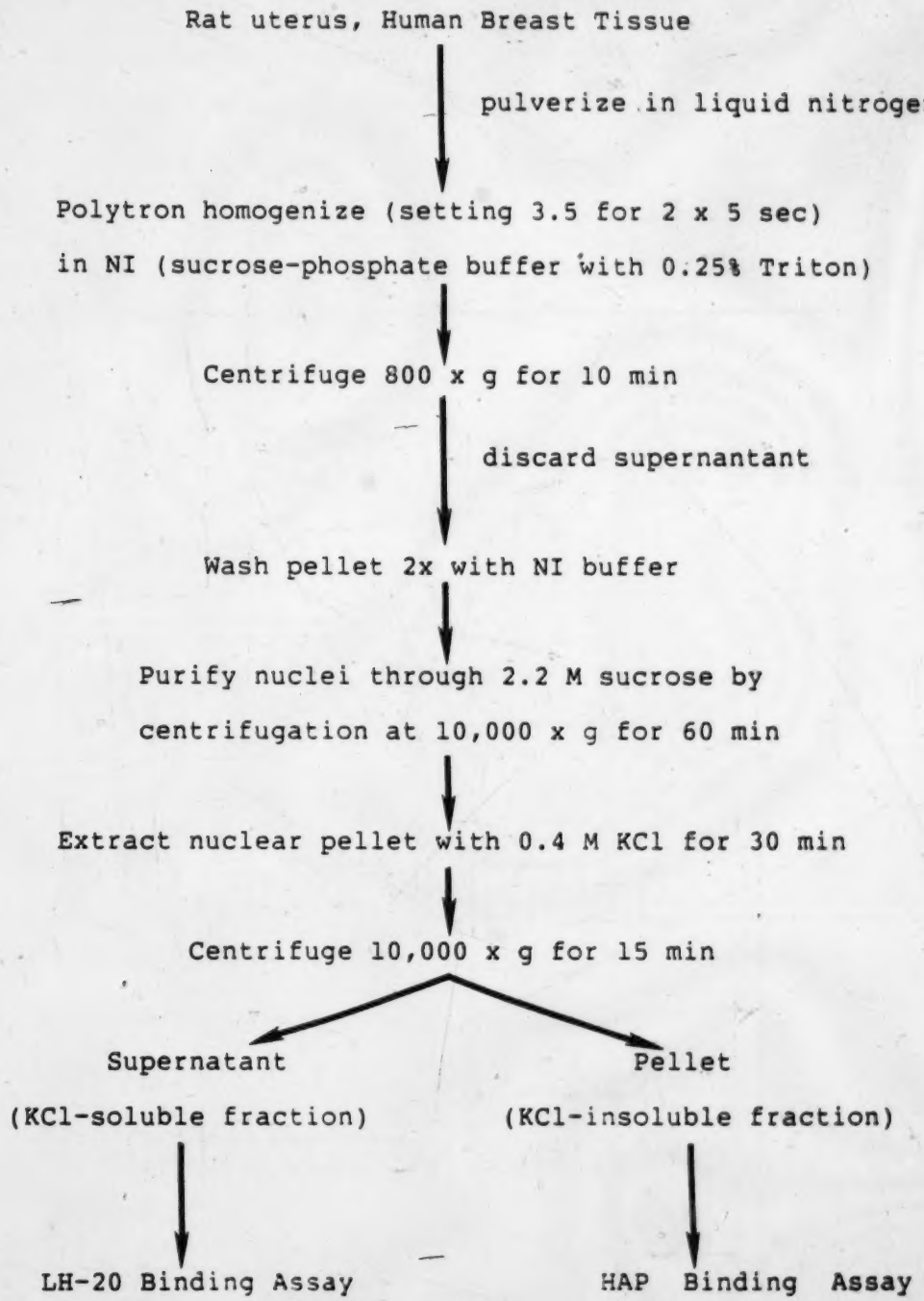
Rat uterus, Human Breast Tissue
pulverize in liquid nitrogen
Polytron homogenize (setting 3.5 for 2 x 5 sec)
in NI (sucrose-phosphate buffer with 0.25% Triton)
Centrifuge 800 x g for 10 min
discard supernantant
Wash pellet 2x with NI buffer
Purify nuclei through 2.2 M sucrose by
centrifugation at 10,000 x g for 60 min
Extract nuclear pellet with 0.4 M KCl for 30 min
Centrifuge 10,000 x g for 15 min
Supernatant
(KCl-soluble fraction)
Pellet
(KCl-insoluble fraction)
LH-20 Binding Assay
HAP Binding Assay

Figure 5. Flow diagram for the preparation and assay of the nuclear fraction

(ii) KCl extraction. Sucrose-purified nuclear pellets were suspended in a hypotonic (TD) buffer and nuclear estradiol-receptor complexes were extracted by addition of an equal volume of TD buffer containing 0.8 M KCl. The mixture was kept at 0-4°C for 30 min with occasional stirring on a vortex mixer and was then centrifuged at 10,000 x g for 10 min at 4°C to yield a KCl-soluble supernatant fraction and a KCl-insoluble pellet.

e) Binding Assays

Aliquots of both KCl-soluble and KCl-insoluble nuclear fractions were incubated with tritiated estradiol at 4°C overnight as described for cytosol fractions in Chapter II. To determine the amount of estradiol binding in the nuclear fraction, estradiol, complexed to receptors as a result of 'in vivo' injection, was exchanged with [^{3}H]estradiol by incubating the samples for 30 min at 37°C (exchange conditions, Anderson et al., 1972a). Samples were then cooled at 4°C for 30 min prior to the separation of free [^{3}H]estradiol from estradiol-receptor complexes.

The pellet remaining after KCl treatment (KCl-insoluble fraction) was resuspended in Tris-MoO_4 buffer and incubated with HAP prior to the addition of steroid. Non-specifically bound and free [^{3}H]estradiol in the KCl-soluble fraction and in the KCl-insoluble fraction were removed by adsorption on Sephadex LH-20 and by repeated washing of HAP-adsorbed [^{3}H]estradiol-receptor complex, respectively, as described

in Chapter II. Receptor concentration was determined by Scatchard analysis and expressed as fmol/uterus (Chapter II).

3. RESULTS

a) Estradiol-Binding Capacity of Untreated Immature Rat Uteri

The specific estradiol-binding capacity of uterine tissue from immature rats was determined using the method of Roy and McEwen (1977) as described in the Materials and Methods section. Table 3 shows that all of the estradiol-binding sites were partitioned in the nuclear fraction and that approximately half of these sites could be extracted with KCl. The total amount of receptor, 1226±212 (mean±S.D.) fmol/uterus, was similar to the amount of receptor observed in experiments where specifically bound estradiol in the cytosol fraction was determined by DCC assay (Chapter II).

b) Influence of Estradiol on Estrogen Receptors of Immature Rat Uteri

Estradiol treatment appeared to result in an increase in the total number of specific estradiol-binding sites in the nuclear fraction of immature rat uteri (Table 3). This number was significantly higher ($t=9.37$, $p<0.05$) than the number of specific estradiol-binding sites observed in the

Table 3

Estradiol-binding capacity of immature rat uteri.

	Concentration of estrogen receptors fmol/uterus (mean±S.D.)				
	Control	Estradiol-primed			
Cell Fraction	(n=4)	(n=4)	(n=3)	(n=3)	(n=3)
Cytosol	no binding	-------	no binding	----------	
KCl-soluble	598±168	566±50	638±105	484±81	673±103
KCl-insoluble	628±143	696±59	1006±75	1620±137	1091±151
Total [ER]	1226±212	1262±56	1644±154	2104±158	1764±187

Weights of immature rat uteri ranged from 0.25-0.30 mg.

Mean rat uterine weights for individual batches were determined from the weights of twelve uteri.

Tissue powders were homogenized in NI buffer (see Materials and Methods); thus this control differs from that of Table 2.

cytosol fraction (see Table 2). For thirteen determinations on four groups of animals, the average number of specific nuclear estradiol-binding sites per immature rat uterus, 30 min after injection of 10 µg of estradiol, was 1693±509 fmol (Table 3). The concentration of estrogen receptors of uteri of immature rats observed in this study was consistent with the receptor levels observed by other investigators (Clark and Peck, 1976; Gannon and Gorski, 1976; deBoer et al., 1977; Schoenberg and Clark, 1980; Markaverich et al., 1981; Jordan et al., 1985) whose results ranged from 985-1700 fmol/uterus.

c) Estradiol-Binding in KCl-Soluble and KCl-Insoluble Nuclear Fractions of Estradiol-Primed Immature Rat Uteri

Also shown in Table 3 is the amount of specific estradiol-binding in the KCl-soluble and KCl-insoluble nuclear fractions. The amount of specifically bound estradiol observed in the KCl-soluble fraction represented a mean of 34% of the total amount of specific nuclear estradiol-binding. The remaining 66% represented the amount of receptor that remained resistant to salt treatment.

d) Influence of Homogenization Buffer on Cytosolic/Nuclear Distribution of Estrogen Receptors of Untreated Immature Rat Uteri

In experiments designed to determine the influence of

the homogenization buffer on the intracellular localization of estrogen receptors, it was found that Tris-molybdate buffer (used in the preparation of cytosols), was effective in solubilizing unoccupied receptor into the cytosolic compartment (Table 4). Of the total estradiol-binding sites, 74% were partitioned in the cytosolic fraction, and the remaining 26% could be extracted from the nuclear pellet. This observation was consistent with the results reported by Carlson and Gorski (1980) who demonstrated that 89% of estrogen binding sites were solubilized into the cytosol fraction of uteri of 21-24 days-old untreated rats, while 11% remained in the nuclear pellet.

Sodium molybdate results in receptor aggregation and thus inhibits receptor transformation and subsequent binding of the estradiol-receptor complex to DNA cellulose (Muller et al., 1983). Consequently, although KCl itself can result in receptor activation and binding to DNA (Muller et al., 1983), the inhibitory effect of molybdate on the transformation reaction may explain why no KCl-insoluble estrogen-binding sites were observed in uterine nuclei that had been homogenized in Tris-MoO_4 buffer. Furthermore, when uteri from estradiol-primed immature rats were homogenized in Tris-molybdate buffer (data not shown), we found that this buffer could no longer solubilize occupied estrogen receptors into the cytosolic compartment.

Table 4

Influence of homogenization buffer on cytosolic/nuclear distribution of estrogen receptors of untreated immature rat uteri.

	Homogenization Buffer*	
	NI	Tris-MoO_4
Cell Fraction**	Estrogen receptor concentration fmol/uterus (mean±S.D.)	
Cytosol	no binding (n=4)	1061±128
KCl-soluble	598±168	368±129
KCl-insoluble	628±143	no binding
Total [ER]	1226±212	1429±65

* See Buffers under Materials and Methods

** See Materials and Methods

e) Assay Variability

The range of nuclear receptor content observed between batches of powdered uteri (Table 3) was consistent with the range observed for cytosolic receptor content (Chapter II). The coefficient of variation in estradiol-binding capacity observed within each batch of powders (intra-assay) was 8% and between batches (inter-assay) the coefficient of variation was 30%. The high inter-assay variation observed in KCl-soluble and KCl-insoluble fractions may, in part, be related to the small sample size or to the age of the rats and levels of endogenous hormones. However, a major source of variation was likely due to the change in the physical characteristics of the nuclear fraction upon exposure to 0.4 M KCl. The high ionic strength buffer caused the nuclear material to become very gelatinous and fibrous, perhaps protecting receptors from extraction.

Initial experiments to determine the DNA content of the rat uteri were carried out on the entire pellet that remained following exposure to KCl (Burton, 1955). However, when the KCl-insoluble pellet was resuspended in buffer for the determination of receptor concentration, the consistency of the KCl-insoluble pellet was such that homogeneous aliquots and accurate DNA determinations were quite impossible to obtain. A brief polytron homogenization (Polytron PT-10) of the salt-insoluble fraction to overcome this problem was unsuccessful and the results were more consistent when the receptor concentration was expressed as

fmol/uterus. The KCl-insoluble pellets were also incubated with DNase I in an attempt to make the pellets more manageable. However, perhaps because of the small quantity of nuclear pellet, this procedure still did not yield homogeneous aliquots.

4. DISCUSSION

a) Estradiol-Binding Capacity of Rat Uterus

The variation of receptor content observed in preparations of rat uteri was discussed in detail in Chapter II. For example, the number of nuclear estradiol-binding sites in uteri of untreated rats may reflect the quantity of endogenous estrogen that is present in the immature animal. Exposure to a pharmacological dose of estradiol appears to result in a increase in the total number of estradiol-binding sites. Pilot studies, comparing the effect of LH-20 and HAP assays on the quantification of nuclear estrogen receptors, indicated that the increased number of receptors was not due to these assay techniques. Although the LH-20 assay could not be used to quantify receptors in the gelatinous KCl-insoluble fraction, both LH-20 and HAP assays were equally effective for the quantification of nuclear estrogen receptor in the KCl-soluble fraction (results not shown).

Estradiol, which is responsible for the characteristic physiological changes in estrogen-sensitive tissues, could

promote an increase in receptor synthesis and thus result in an increase in the total number of binding sites in estrogen target tissues. But, this increase in receptor content would not happen in the 30 minutes during which the animals are exposed to a pharmacological dose of estradiol (Anderson 1972b; Clark et al., 1972). However, a chronic exposure to low concentrations of estadiol in some animals could account for higher nuclear levels of receptor (Cidlowski and Muldoon, 1974; Zava et al., 1976). Another explanation for the apparent increase in the concentration of cellular estrogen receptors following estradiol injection has been proposed by Garola and McGuire (1977b). These investigators attributed the increase to protection of the receptor by estradiol during homogenization and fractionation procedures.

b) Importance of Buffers for the Assay of Nuclear Estrogen Receptors

There is considerable evidence which indicates that localization of unoccupied estrogen receptor sites in the nuclear fraction of uteri of untreated immature rats does not arise as a result of cytoplasmic estrogen receptor becoming trapped in the nuclear pellet as a result of homogenization (Panko and MacLeod, 1978). First, as shown by other workers (Carlson and Gorski, 1980), when uteri of untreated immature rats were homogenized in the presence of high cytosolic estrogen receptor levels, only a small

increase in estrogen receptor levels was subsequently measured in the nuclear fraction of these uteri. Thus, Carlson and Gorski (1980) concluded from their experiments that estrogen receptors were not randomly trapped by a large number of non-specific sites in the nuclear pellet.

Second, the inclusion in the homogenization buffer (NI) of 0.25% Triton X-100, which strips away the outer plasma membrane and is able to freely penetrate the nuclear membrane (Smuckler et al., 1976) did not reduce the total estradiol-binding capacity of rat uteri. This result suggests that estrogen receptors are not associated with the nuclear pellet through hydrophobic bonding.

Third, nuclear estrogen-binding sites probably do not arise as a result of chemical transformation of cytoplasmic receptor and subsequent translocation to the nucleus. The evidence for this comes in part from the studies of Fukai amd Murayama (1984) who observed that concentrations of up to 10 mM Mg^{++} had no effect on nuclear translocation of of cytosolic estrogen receptors. In addition, the concentration of 0.25% Triton X-100 and 3 mM Mg^{++}, which are present in the homogenization buffer, has been reported to result in degradation of cytosolic estrogen receptors in the absence of nuclei (Roy and McEwen, 1977).

Fourth, purification of nuclei through 2.2 M sucrose resulted in very little contamination with cytoplasmic debris when the tissue was homogenized in NI buffer.

Fifth, the observation that 0.4 M KCl could extract one half of the total nuclear receptor content in untreated rat uteri suggests that unoccupied receptor-chromatin interaction is in part, due to ionic binding.

All of the above data are consistent with the current hypothesis that steroid receptors in target tissues are predominantly nuclear and can be artifactually redistributed to the cytosol during tissue processing. Unoccupied estrogen receptors can be easily solubilized and separated from the particulate components of untreated immature rat uteri. However, an injection of estradiol quickly results in high affinity binding of estradiol-receptor complexes to chromatin acceptor sites. Receptor sites occupied by endogenous estrogens cannot be readily solubilized, but appear to be differentially extracted by 0.4 M KCl.

c) KCl-Soluble and KCl-Insoluble Estrogen Receptors

Attempts to gain a better understanding of the mechanism of steroid action have been directed towards defining the relationship between steroid-receptor complexes and nuclear acceptor sites. Clark and Peck (1976) were the first to demonstrate that the number of KCl-insoluble receptors in uteri of estradiol-primed immature rats was approximately equal to the number of sites required for true uterine growth and suggested that it was these sites that were physiologically the most important.

Several other investigators have also observed KCl-insoluble sites in uterine nuclei of immature rats; however, there is considerable disagreement regarding the proportion of KCl-soluble to KCl-insoluble sites. For instance, some investigators claim extraction percentages of 80-90% (Juliano and Stancel, 1976; Muller et al., 1977), whereas, others are able to extract only 35-55% of nuclear estradiol-binding sites (Ruh and Baudendistal, 1977; Ruh et al., 1977; Barrack and Coffey, 1982; Katzenellenbogen et al., 1978; Attardi, 1983). In the present study, the results were more in agreement with the results of Ruh and colleagues than with Juliano and colleagues.

Several methodological differences have been suggested as explanations of the contradictory results for extraction of receptors from rat uteri (Ikeda et al., 1982). Ikeda observed that a single extraction of rat uterine nuclei with 0.4 M KCl 1 or 6 hr after 'in vivo' injection of [^{3}H]estradiol (direct assay) removed 80-85% of the total nuclear bound [^{3}H]estradiol. Two additional extractions removed 95% of the total nuclear bound [^{3}H]-estradiol.

However, when the KCl-extractability of the estradiol-receptor complex was determined using [^{3}H]estradiol exchange assay (immature rats were injected with unlabelled estradiol), the nuclear bound [^{3}H]estradiol in the KCl-soluble fraction was reduced to 60-70% of the total amount. In addition, the proportion of KCl-soluble to KCl-insoluble

binding sites could be further manipulated, depending on whether the [^{3}H]estradiol exchange assay was performed before or after KCl extraction of the nuclei (Ikeda et al., 1982).

The most significant factors affecting receptor extractability appeared to be conditions used for extraction of receptors and for the exchange assay (Ikeda et al., 1982), as well as the osmolarity and constituents of solutions used for assays of nuclear receptor (Ruh and Baudendistal, 1977). The considerable variation in the ratio of KCl-soluble to KCl-insoluble estrogen receptor, reported in the literature, leaves little doubt that experimental conditions are critical to the evaluation of the characteristics of nuclear binding of estradiol-receptor complex. Thus it remains to be established whether two unique receptor populations, a KCl-soluble and a KCl-insoluble receptor population, exist 'in vivo'.

d) Receptor Stability

The longer period of cold storage of uteri used in this study (batches 1 and 2 were stored 4-6 months prior to receptor assay as opposed to 1-2 months for batches 3 and 4) may have resulted in a small loss of receptor concentration, however, receptor concentrations were within the expected range of values. Controlled studies to determine the stability of nuclear estrogen receptor of rat uteri stored at -70^{o}C were not carried out, but others

have reported that estrogen receptor remains stable at -70°C for several months (Wittliff et al., 1980; Hyder and Leake, 1982).

5. CONCLUSIONS

Comparison of the quantity of specific estradiol-binding sites in uteri of estradiol-primed rats with the quantity of estradiol-binding sites in uteri of untreated rats led to the following conclusions:

i) Exposure of immature rat uteri to estradiol results in an apparent increase in the number of specific nuclear estradiol-binding sites.

ii) Estradiol-binding sites are not degraded during nuclear assay procedures used.

iii) Estradiol, previously complexed with nuclear estradiol-binding sites as a result of hormone injection, apparently exchanges with [^{3}H]estradiol during incubation of the nuclear fraction at 37°C.

Therefore, nuclear assay procedures used in this study can be used to accurately determine the number of specific nuclear estradiol-binding sites in the estrogen-sensitive tissue, rat uterus. Consequently, it was assumed that this method could be useful for quantification of nuclear estradiol-binding sites in both normal and neoplastic estrogen target tissues during the estrous cycle.

CHAPTER IV

NUCLEAR AND CYTOSOLIC ESTROGEN RECEPTORS AND PROGESTERONE RECEPTORS IN MALIGNANT BREAST TISSUE

1. INTRODUCTION

A sensitive assay for the determination of the nuclear estrogen receptor content of human breast tumors may help to explain why 25% of tumors containing estrogen and progesterone receptors fail to regress when exposed to endocrine therapy and why 5-10% of tumors which apparently lack receptors regress in response to endocrine therapy. Such a nuclear assay may provide a more accurate correlation between receptor status and clinical response.

Having validated assay techniques for the quantification of nuclear estrogen receptors in rat uterus, the methods were applied to samples of human breast tumors. Quantifying nuclear estradiol-binding sites in human breast tumor samples for the purpose of determining the relationship between the incidence of nuclear and cytosolic estrogen receptors and progesterone receptors was the final research objective of this project.

2. METHODS

a) Tissue

Samples of human breast tumor tissue were obtained from portions of frozen specimens (stored at -70°C) that remained following routine clinical assays for cytosolic estrogen receptors and progesterone receptors. The criteria for selection of consecutively numbered specimens was based on tissue weight (0.25-5.0 g) to ensure an adequate amount of material for incubation with a sufficient number of steroid concentrations in order to perform Scatchard analysis of the binding data. Tissues were powdered in liquid nitrogen and stored at -70°C until required.

Rat uterus was used as a reference powder for the determination of nuclear estrogen receptor content.

b) Binding Assay

The nuclear assay for estrogen receptor, as validated previously (Chapter III), was applied to 34 human breast tumor samples. The samples were confirmed to be malignant by checking the pathology report. The estrogen receptor status of the nuclear fraction was then compared to the estrogen and progesterone receptor status, which had been previously determined for the cytosolic fraction of the same tissue.

3. RESULTS

Samples of breast cancer tissue from 34 patients were assayed for nuclear estradiol-binding sites using techniques described previously (Chapter III). Nuclear estrogen receptors could be detected in approximately one half of the samples. (The data obtained are shown in Appendices I and II.) Nuclear estradiol-binding sites were those sites which could be extracted from nuclei using 0.4 M KCl; none of the human breast tumor samples analyzed in this preliminary study were found to contain KCl-insoluble nuclear estrogen receptors.

The relationship between concentrations of nuclear and cytosolic estrogen receptors and progesterone receptors is shown in Table 5. Among the 34 samples, 19/34 (56%) contained both cytosolic estrogen and progesterone receptors, 13/34 (38%) contained cytosolic and nuclear estrogen receptors, and 13/34 (38%) contained all three receptors.

The distribution of nuclear estrogen receptors and/or progesterone receptors was analyzed by the test of proportions. Both types of receptors were present more frequently in tumors containing cytosolic estrogen receptors than in tumors lacking cytosolic estrogen receptors. More than half of the samples (13/22) which contained cytosolic estrogen receptors also contained nuclear estrogen receptors ($z=2.49$, $p<0.05$), and a significant proportion (19/22)

Table 5

Frequencies of the presence of cytosolic progesterone receptors (PgR) and nuclear estrogen receptors (ERn) at various concentration ranges of cytosolic estrogen receptors (ERc) in human breast tumors.

Receptor Status	Concentration of cytosolic estrogen receptors (fmol/mg cytosol protein) <10	10-100	>100	Total
PgR +ve	1/12	7/9	12/13	20/34
ERn +ve	3/12	3/9	10/13	16/34
PgR +ve, ERn +ve	1/12	3/9	10/13	14/16

<10 fmol/mg of cytosol protein is defined as cytosolic ERc/PgR-negative.

<25 fmol g of tissue is defined as ERn-negative. The cut-off value for ERn is a measure of the sensitivity of the assay.

PgR and ERn were present more frequently in ERc +ve tumors than in ERc -ve tumors (PgR: 19/22 vs 1/12, $z=8.12$, $p<0.05$; ERn: 13/22 vs 3/12, $z=2.49$, $p<0.05$).

contained cytosolic progesterone receptors ($z=8.12$, $p<0.05$). Also, the incidence of tumors containing all three receptor populations was found to increase as the concentration of cytosolic estrogen receptors in the tumors increased (3/9 vs 10/13, $z=2.02$, $p<0.05$).

Three tumors which had no measureable cytosolic estrogen receptors (3/12) contained nuclear estrogen receptors and only one of the tumors (1/12) contained both progesterone receptors and nuclear estrogen receptors (Table 5).

The majority of tumors containing nuclear estrogen receptors (14/16) also contained progesterone receptors (Table 6).

The quantitative relationship between cytosolic estrogen receptor content and nuclear estrogen receptor content is shown in Table 7. The cut-off value for nuclear estrogen receptors of 25 fmol receptor/g wet weight of tumor was based on similar studies on nuclear estradiol receptors in human mammary carcinoma (O'Connell et al., 1982) and is more a measure of the sensitivity of the assay rather than a reflection of the receptor content required to elicit a response to endocrine therapy. Because the binding data of the cytosol fraction was not calculated as fmol/g of tissue, a true comparison of this data with the binding data of the nuclear fraction was not possible; however, based on studies using rat uteri (Chapter III), it was expected that all of the receptors that were measured in the nuclear fraction represented the total estrogen receptor content of the cell

Table 6

Presence of cytosolic progesterone receptors (PgR) and nuclear estrogen receptors (ERn) in 34 human breast tumors.

	PgR negative *	PgR positive
nuclear ER-negative **	12	6
nuclear ER-positive	2	14

* <10 fmol/mg of cytosol protein is defined as progesterone receptor-negative.

** <25 fmol/g of tissue is defined as nuclear estrogen receptor-negative

$\chi^2 = 8.2$

Table 7

Quantitative relationship between nuclear estrogen receptors and cytosolic estrogen receptors of 34 human breast tumors.

Nuclear ER (fmol/g tissue)	Cytosolic ER (fmol/mg cytosol protein)		
	<10 *	10-100	>100
<25 **	9	6	3
25-100	2	3	0
>100	1	0	10

* <10 fmol/mg of cytosol protein is defined as cytosolic ER-negative.

** <25 fmol/g of tissue is defined as nuclear ER-negative.

Nuclear ER were present more frequently in [ER>100]-positive tumors than in [ER=10-100]-positive tumors (10/13 vs 3/9, $z=2.02$, $p<0.05$).

and indeed, there was a positive relationship between the nuclear estrogen receptor content and the cytosolic estrogen receptor content ($z=2.02$, $p<0.05$).

The frequency of nuclear and cytosolic estrogen receptors and progesterone receptors relative to menstrual status is shown in Table 8. There was a tendency for tumors containing cytosolic estrogen receptors to be more prevalent in postmenopausal women (18/24) than in premenopausal women (4/10) ($z=1.69$, $p<0.05$) and receptor-positive tumors in older women had higher levels of cytosolic estrogen receptors.

As there was a positive relationship between cytosolic estrogen receptor content and the presence of both progesterone receptors and nuclear estrogen receptors (see above), there was a significantly higher percentage of cytosolic estrogen receptor-positive tumors containing both progesterone receptors and nuclear estrogen receptors from postmenopausal patients than from premenopausal patients (11/24 vs 2/10 respectively; $z=2.28$, $p<0.05$).

Table 8

Frequency of the presence of progesterone receptors (PgR) and/or nuclear estrogen receptors (ERn) relative to cytosolic estrogen receptors (ERc) and patient menstrual status in human breast tumors.

	Menstrual Status				
	Premenopausal		Postmenopausal		
	ERc -ve (n=6)	ERc +ve (n=4)	ERc -ve (n=6)	ERc +ve (n=18)	#/Total
PgR +ve	1/6	4/4	0/6	15/18	(20/34)
ERn +ve	2/6	1/4	1/6	11/18	(15/34)
+ve/+ve	1/6	2/4	0/6	11/18	(14/34)

<10 fmol/mg of cytosol protein is defined as cytosolic estrogen (ERc) and progesterone receptor (PgR)-negative

<25 fmol/g wet weight of tissue is defined as nuclear estrogen receptor (ERn)-negative

There was a tendency for ERc to be more prevalent in tumors from postmenopausal patients (18/24) than in tumors from premenopausal patients (4/10) ($z=1.69$, $p<0.05$).

The number of tumors which contained all three receptor populations was significantly higher in postmenopausal patients (11/24) than in premenopausal patients (2/10) ($z=2.28$, $p<0.05$).

4. DISCUSSION

a) Validity of nuclear assay techniques

Despite the fact that two unique nuclear estrogen receptor populations could be detected in uteri of estradiol-primed immature rats, no KCl-insoluble receptors were detected in the nuclear fraction of human breast tissue. Some authors claim that KCl-insoluble receptors represent the biologically active form of the receptor (Clark and Peck, 1976), whereas others doubt their existence (see Chapter III). Discrepancies in the reported ratios of KCl-soluble to KCl-insoluble estrogen receptors in animal models have been explained on the basis of methodological differences in nuclear assay techniques (Ikeda et al., 1982).

There are several techniques available for measuring the estrogen receptor content of target tissues; however, an ideal assay, one that is relatively fast, yet inexpensive, is not yet available (Chamness and McGuire, 1979; Leake, 1981). Differences in methodology and subsequent handling of data are the major sources of variation in quantitative estimates of receptor content of target tissues (Wittliff, 1984). Therefore, the initial objective of this study was to ensure that the methodology used for nuclear estrogen receptor analysis would result in reproducible data. One main source of variation in estimates of receptor concentration has been attributed to tissue heterogeneity, particularily in tumor tissue (Braunsberg, 1975;

Silfversward et al., 1980). This problem was minimized by preparing homogeneous powders.

The assay results of the nuclear estrogen receptor content of rat uteri were in good agreement with those of other authors (see Chapter III) and indicated that there were no methodological factors related to the Sephadex LH-20 or HAP techniques, or to the nuclear assay itself, that would explain why no KCl-insoluble nuclear estrogen receptors could be detected in human breast tumors.

Studies of the estrogen receptor content of target tissues of animal models are based mainly on single hormone injections to hormone-depleted animals. The growth of breast epithelium has been found to be influenced by estrogens, progestins, glucocorticoids, androgens, prolactin, insulin, and growth hormone (Leake, 1984). Therefore, hypotheses derived from experimental models cannot be readily applied to the 'in vivo' situation where hormones are continually secreted and their effects modified by complex interaction with other circulating hormones and/or other factors. However, by using rat uterine tissue as an internal control for the assessment of assay performance, it was concluded that the nuclear estrogen receptor content of human breast tissue could be accurately quantified.

b) Receptors and Response to Therapy

The final research objective of this project was to determine if a relationship existed between nuclear estrogen receptors and cytosolic estrogen and progesterone receptors of human mammary tumors. In the four classifications of tumors, based on cytosolic receptor status, the distribution of cytosolic estrogen and progesterone receptors in the tumor samples that were tested in this study was similar to that reported by the Hormone Receptor Laboratory in a series of 100 consecutive samples (ER+ve, PgR+ve = 55%; ER-ve, PgR-ve = 27%; ER+ve, PgR-ve = 8%; ER-ve, PgR+ve = 10%). Thus it was concluded that the tumors assayed in this study were fairly representative of the tumor population in general.

Several groups have stressed the importance of identifying a "functional" estrogen receptor system (see Chapter I) for the assessment of the hormonal sensitivity of human breast cancer. The results of this preliminary study of the incidence of cytosolic and nuclear estrogen receptors and progesterone receptors in human breast tumors may be useful in identifying more accurately those patients who could benefit from endocrine therapy.

The determination of the nuclear estrogen receptor status of [illegible] tumor specimens was originally proposed by Laing et al. (1977) in an effort to identify an intact receptor system. It was thought that the presence of

estrogen receptors would identify "false-positives" (those patients whose tumors contained estrogen receptors but failed to respond to endocrine treatment). Laing suggested that false-positive measurements resulted from a breakdown in the translocation process. However, the presence of nuclear estrogen receptors alone would not indicate a "functional" receptor system as proposed by Laing et al. (1977) because the mere presence of nuclear estrogen receptors does not preclude the possibility of a defect in the receptor such that it can no longer bind estrogens (Thorsen and Stoa, 1979). Additionally, an inability of estradiol-receptor complexes to bind to the appropriate DNA sites (Sato et al., 1981; Taylor et al., 1980), or a breakdown at the post-transcriptional level at any one of the steps that initiate mRNA and protein synthesis could occur even in the presence of estrogen-receptor complexes.

Although "translocation" is now recognized as an artifact by most workers (Jensen, 1984; Walters, 1985), there is evidence to suggest that as many as 20% of breast cancer patients have tumors containing estrogen receptors that fail to undergo activation or to bind to chromatin acceptor sites (Leake, 1984; Fazekas and MacFarlane, 1983; Hawkins et al., 1983). Defective receptors might be equivalent to unoccupied sites and therefore be more readily solubilized into the cytosol fraction as compared to intact occupied receptors. If this were true for this group of

the absence of nuclear estrogen receptors would probably mean that the tumor was one which would not likely respond to endocrine treatment.

Based on the criteria that both cytosolic and nuclear estrogen receptors would be required in order for the tumor to be responsive to endocrine therapy, our results allow us to hypothesize that a response could be predicted for 13/34 (38%) rather than for 22/34 (64%) patients if cytosolic estrogen receptor status alone is considered. Similarily, if the induction of progesterone receptor synthesis requires a functional nuclear estrogen receptor, then the presence of both nuclear estrogen receptors and progesterone receptors in tumors would indicate an intact receptor mechanism that would be responsive to therapy. Thus, hypothetically, a response could be predicted for 14/34 (41%) rather than for 19/34 (56%) patients based only on cytosolic estrogen and progesterone receptor status. If it is assumed that the presence of all three receptors would provide a more accurate indicator of hormonal dependence of tumors, a response could be predicted for 13/34 (38%) patients.

Thus, it was concluded from the data that the presence of nuclear estrogen receptors and progesterone receptors in breast tumor samples might provide a more accurate indication of hormone-sensitive tumors. In one study (Barnes et al., 1979), the data from triple steroid receptor assays suggested that the response rate might indeed be slightly

further clinical trials.

c) Receptors and Menstrual Status

Patients can be divided into groups with respect to cytosolic estrogen receptor levels and age or menstrual status. The age groups of <50 and >50 years are usually equated, for convenience, with premenopausal and postmenopausal states, respectively (McGuire et al., 1975b). In practice however, age, which may be the more important factor (Elwood and Godolphin, 1980), does not necessarily reflect the menstrual status. In our study, one patient >50 was found to be premenopausal.

Many authors have found that breast cancer tissue from postmenopausal women contains higher levels of cytosolic estrogen receptors than that from premenopausal patients (McGuire et al., 1975b; Allegra et al., 1979; Jensen, 1981; Kinne et al., 1981). The presence of steroid receptors in some tumors from postmenopausal women can be detected because, although circulating levels of steroid hormones are lower, apparently the level of endogenous estrogen is sufficient for receptor activation and induction of progesterone synthesis (Saez et al., 1978).

Although there is a lower incidence of cytosolic estrogen receptors in tumors of premenopausal patients, a lower concentration of receptor can elicit a response to endocrine intervention (Jensen et al., 1975; King et al.,

1985; Rochman et al., 1985). It has been hypothesized that the lower incidence of cytosolic estrogen receptors in tumors from premenopausal patients could be explained by assuming that standard receptor assays were not designed to detect nuclear estrogen receptors. However, the lower incidence of all three receptor populations in tumors from these patients suggests that low estradiol-binding activity cannot be explained by saturation of estrogen receptor sites with endogenous estrogens. It may instead, be a reflection of other hormonal influences. In premenopausal women, circulating levels of estrogens are high during the first half of the menstrual cycle, but decrease during the second half of the cycle when high levels of progesterone, released after ovulation, repress estrogen receptor synthesis (Wittliff, 1984). Therefore, a greater understanding is required of the factors which can influence receptor levels under the dynamic conditions of the normal physiological state.

It has been suggested that these differences observed in the proportion of estrogen receptor-positive tumors and in tumor responses between postmenopausal and premenopausal patients may be associated with the normal aging process, rather than with any of the epidemiological risk factors associated with carcinoma of the breast (Elwood and Godolphin, 1980; Rochman et al., 1985).

d) Menopausal Status and Response to Endocrine Therapy

The response rate that would be predicted from our results, on the basis of all three receptors being present in tumor biopsies, would be 2/10 (20%) premenopausal patients and 10/24 (42%) postmenopausal patients. These predictions, are in close agreement with the observed incidence of patient response reported from large clinical studies (Bertuzzi et al., 1981; McCarty et al., 1983; Leake, 1984). Bertuzzi and colleagues reported that functional estrogen receptors in tumor specimens from premenopausal patients were found in 26% of the cases and in 42% of postmenopausal patients.

If one assumes a response to endocrine therapy in patients with a functional or intact receptor mechanism, the predicted response rates, based on the three receptor measurements, can be compared with those based on the presence of cytosolic estrogen and progesterone receptors only, as determined by routine receptor assays. In the latter case, a higher, but not more accurate response rate would be predicted, namely, 4/10 (40%) premenopausal patients and 15/24 (63%) postmenopausal patients.

The actual response rate to endocrine therapy of breast cancer patients is known to be about 25% in unselected premenopausal women and about 35% in postmenopausal women (McGuire et al., 1982; Leake, 1984). Thus, it can be seen from the above data that a hypothetical response rate closer

to the actual observed response rate can be predicted when the status of all three receptors is used.

The hypothesis that pre- and postmenopausal disease may have different biological characteristics is supported by the bimodal age distribution of estrogen receptors in breast tumor samples and response to endocrine therapy (de Waard, 1969; Paffenbarger et al., 1980; Devitt, 1982). Further support for this hypothesis has come from recent histochemical methods (King et al., 1985; Pertschuk et al., 1978; 1985). These methods, which localize estrogen receptor in human breast tumors by intensity of staining patterns, revealed differences between tissue obtained from pre- and postmenopausal patients (King et al., 1985). The less intense staining patterns generally observed in tumors from premenopausal women suggest that low cytosolic steroid-binding activity is not due to a masking of receptors by endogenous estrogens and that other factors are responsible.

e) Relationship of Progesterone Receptors and Nuclear Estrogen Receptors

The results reported in this study could also explain why a small group (5-10%) of patients with estrogen receptor-negative tumors consistently respond to endocrine therapy. A small proportion of samples (3/12) contained nuclear estrogen receptors in the absence of cytosolic estrogen receptors and only 1 of the 12 (8%) tumors, negative for cytosolic estrogen receptors, contained both

nuclear estrogen receptors and progesterone receptors.

Although synthesis of progesterone receptors can occur in some tumors independent of the estrogen-receptor mechanism (Allegra et al., 1979), the inclusion of progesterone receptor determinations in routine assays has dramatically increased the predictive index of estrogen receptor status (McGuire et al., 1975b). Therefore the presence of both progesterone receptors and nuclear estrogen receptors may provide a significant indication of a "functional" receptor system that would be sensitive to endocrine treatment.

Based on the current model of steroid-cell interaction, it would be expected that the absence of progesterone receptors in some tumors containing estrogen receptors would be due to increased affinity of progesterone receptors for nuclear components (Mockus and Horwitz, 1983). Therefore, additional work to develop a nuclear assay for progesterone receptor may prove useful.

f) Current Status of Breast Cancer Treatment and Receptor Assays

Although breast cancers represent a variety of pathological states arising from multiple factors, the effect of age or menstrual status is now recognized as being "sufficiently distinct to generally permit substantially different therapy for pre- and postmenopausal women,

regardless of the receptor status" (Lippman, 1985) The NIH Consensus Development Panel (Lippman, 1985), reported that a substantial reduction in mortality was achieved by treating premenopausal women with cytotoxic chemotherapy, regardless of hormone receptor status. The Panel also reported that for most postmenopausal women, regardless of receptor status, the antiestrogen drug, Tamoxifen, "improved the recurrence-free survival time equally in patients with or without axillary node involvement" (Lippman, 1985). However, the Panel failed to address the issue of combination endocrine and chemotherapy, and since many experts in the field of breast cancer treatment are unable to agree upon optimal therapies (Lippman, 1985), a nuclear assay for the determination of receptor status may still enable the clinician to select the best form of therapy.

Unfortunately, the determination of both cytosolic and nuclear receptors is time consuming and requires a considerable amount of tissue, which is often not available. Therefore, better assays which are not as demanding and which can be carried out on smaller amounts of tissue are preferable. Consequently, production of monoclonal antibodies against the receptor protein (Greene et al., 1980a; 1980b) has permitted the development of estrogen receptor assays based on antigenic recognition rather than steroid binding activity (Jensen et al., 1982). These assays permit a more precise characterization of cell types containing receptors (antigens) and are able to quantify receptors,

regardless of their affinity for chromatin, in a small number of cells, such as are obtained in a needle biopsy. Until these assays have been completely assessed as to their clinical value they can provide a useful adjunct to biochemical assays of receptor.

5. CONCLUSIONS

This preliminary study demonstrated that techniques used to quantify nuclear estrogen receptors in target tissues provided accurate reproducible estimates of receptor content; however, the existence of KCl-insoluble estrogen receptors in human breast carcinoma was not established.

The data presented in this study suggest that the presence of both cytosolic and nuclear estrogen receptors along with cytosolic progesterone receptors may indeed improve the accuracy of predicting response to hormonal therapy, particularly in postmenopausal patients. However, a larger number of samples must be assayed in order to obtain statistically meaningful data, and the clinical correlation between the presence of nuclear estrogen receptors in cytosolic estrogen receptor-negative, progesterone receptor-positive tumors and response to endocrine therapy must be established before the value of nuclear estrogen receptor determinations in tumors of patients with circulating estrogens can be ascertained.

The author hopes this work will generate further interest and research effort in nuclear steroid receptors.

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APPENDICES

APPENDIX 1

Concentration of steroid receptors in human mammary tumors used in this study. Data are ranked by patient age.

Patient		Receptor Concentration*			
Tumor #	Age	ERc	PgR	ERn	
6442	32	0	11	121	
6449	40	2	4	0	
6444	42	0	8	0	
5440	43	19	29	0	premeno-
6448	45	0	0	0	pausal
5465	46	47	16	36	
5460	47	29	64	0	
6491	47	4	0	0	
6483	49	0	0	55	
5308	51	616	857	176	
6409	51	0	0	51	
5452	52	181	24	128	
5466	56	4	6	0	
6458	56	177	192	0	
6447	60	42	67	97	
6446	60	10	0	22	
6468	61	685	0	0	
5462	62	38	8	17	
6456	62	18	32	0	
5304	63	557	1017	320	postmeno-
5454	65	0	0	22	pausal
6457	66	112	131	230	
5310	68	238	137	373	
6404	68	20	20	69	
6461	76	447	740	534	
5314	77	238	682	1189	
6450	77	3	0	0	
5467	78	7	1	16	
6421	79	216	61	0	
6493	79	332	231	2598	
6451	82	30	35	0	
6492	82	8	0	0	
6493	84	339	191	336	
6462	87	793	496	3060	

*Units for receptor concentrations are fmol/mg of cytosol protein for cytosolic ER and fmol/g of tissue for nuclear (ER).

ERc = cytosolic estrogen receptor
ERn = nuclear estrogen receptor
PgR = cytosolic progesterone receptor

APPENDIX 2

Steroid receptor data for human mammary tumors of Appendix 1 showing corresponding data for rat uterine standards.

Tumor #	Receptor Concentration*				
	ERc	PgR	ERn	ERn of rat uterine standard	
				KCl-soluble	KCl-insoluble
5304	557	1017	320		
5308	616	857	176	1750	6164
5310	238	137	373		
5314	238	682	1189		
6404	20	20	69	2112	5376
6409	0	0	51		
5440	19	29	0		
5450	339	191	336	2599	4864
5452	181	24	128		
5454	0	0	22		
5460	29	64	0	2188	3845
5462	38	8	17		
5465	47	16	36		
5466	4	6	0	2984	3878
5467	7	1	16		
6421	216	61	0		
6442	0	11	121	1643	4544
6444	0	8	0		
6446	10	0	22		
6447	47	67	97	2247	2019
6448	0	0	0		
6449	2	4	0		
6450	3	0	0	3360	4056
6451	30	35	0		
6456	18	32	0		
6457	112	131	230	1923	3637
6458	117	192	0		
6461	447	740	534		
6462	793	496	3060	4322	3695
6468	685	0	0		

6483	0	0	55		
6491	4	0	0	2188	3845
6492	8	0	0		
6493	332	231	2596		

* Units for receptor concentrations are fmol/mg of cytosol protein for cytosolic receptors and fmol/g of tissue for nuclear ER.

ERc = cytosolic estrogen receptor
ERn = nuclear estrogen receptor
PgR = cytosolic progesterone receptor